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(54) Title: PRODUCTION AND USE OF MULTIMERIC HEMOGLOBINS (57) Abstract Multimeric hemoglobin-like proteins are obtained by crosslinking cysteines, natural or artificial, of the component tetramers, or by genetically fusing globin-like domains of one tetramer with those of another, by means of an interdomain spacer sequence. Artificial cysteines are introduced selectively in a single globin-like domain per tetramer to control polymerization.		

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PRODUCTION AND USE OF MULTIMERIC HEMOGLOBINS

5 This application is a continuation-in-part of
Ser. No. 07/780,179, filed November 8, 1991, which is a
continuation-in-part of Ser. No. 07/671,707, filed April
1, 1991, which is the national stage of PCT/US90/02654,
filed May 10, 1990, which is a continuation-in-part of (a)
10 Looker and Hoffman, U.S. Ser. No. 07/374,161, DI-ALPHA AND
DI-BETA GLOBIN LIKE POLYPEPTIDES AND USES THEREFOR, filed
June 30, 1989; (b) Stetler and Wagenbach, U.S. Ser. No.
07/379,116, PRODUCTION OF HUMAN HEMOGLOBIN BY TRANSFORMED
YEAST CELLS, filed July 13, 1989; and (c) Hoffman, Looker,
15 Rosendahl and Stetler, U.S. Ser. No. 07/349,623,
POLYCISTRONIC CO-EXPRESSION OF THE ALPHA- AND BETA-GLOBINS
AND IN VIVO ASSEMBLY OF BIOLOGICALLY ACTIVE, TETRAMERIC
HEMOGLOBIN, filed May 10, 1989; all owned by Somatogen,
Inc.

Cross-Reference to Related Applications

20 Hoffman and Nagai, U.S. Ser. No. 07/194,338,
filed May 10, 1988, now U.S. Patent No. 5,028,588,
presently owned by Somatogen, Inc., relates to the use of
low oxygen affinity and other mutant hemoglobins as blood
substitutes, and to the expression of alpha and beta globin
in nonerythroid cells. Hoffman and Nagai, U.S. Ser. No.
25 07/443,950, filed December 1, 1989, discloses certain
additional dicysteine hemoglobin mutants; it is a
continuation-in-part of 07/194,338. Anderson, et al.,
HEMOGLOBINS AS DRUG DELIVERY AGENTS, Ser. No. 07/789,177,
filed Nov. 8, 1991, discloses use of conjugation of
30 hemoglobins with drugs as a means for delivery of the drug
to a patient.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention is directed to multimeric hemoglobin-like proteins composed of two or more pseudotetramers linked together either by genetic fusion or by chemical crosslinking.

Description of the Background Art

A. Structure and Function of Hemoglobin

Hemoglobin (Hgb) is the oxygen-carrying component of blood. Hemoglobin circulates through the bloodstream inside small enucleate cells called erythrocytes (red blood cells). Hemoglobin is a protein constructed from four associated polypeptide chains, and bearing prosthetic groups known as hemes. The erythrocyte helps maintain hemoglobin in its reduced, functional form. The heme iron atom is susceptible to oxidation, but may be reduced again by one of two enzyme systems within the erythrocyte, the cytochrome b₅ and glutathione reduction systems.

The structure of hemoglobin is well known. We herewith incorporate by reference the entire text of Bunn and Forget, eds., Hemoglobin: Molecular, Genetic and Clinical Aspects (W.B. Saunders Co., Philadelphia, PA: 1986) and of Fermi and Perutz "Hemoglobin and Myoglobin," in Phillips and Richards, Atlas of Molecular Structures in Biology (Clarendon Press: 1981).

About 92% of the normal adult human hemolysate is Hgb A (designated alpha₂ beta₂, because it comprises two alpha and two beta chains). Other recognized hemoglobin species are Hgb A₂ ($\alpha_2 \delta_2$), Hgb A_{1a}, Hgb A_{1b}, and Hgb A_{1c}, as

well as the rare species Hgb F (α_2 γ_2), Hgb Gower-1 (ζ_2 ϵ_2), Hgb Gower-2 (α_2 ϵ_2), Hgb Portland (ζ_2 γ_2), and Hgb H (β_4) and Hgb Bart (γ_4). They are distinguished from Hgb A by a different selection of polypeptide chains.

The primary structure of a polypeptide is defined by its amino acid sequence and by identification of any modifications of the side chains of the individual amino acids. The amino acid sequences of both the alpha and beta globin polypeptide chains of "normal" human hemoglobin is given in Table 1. Many mutant forms are also known; several mutants are identified in Table 400. The wild-type alpha chain consists of 141 amino acids. The iron atom of the heme (ferroprotoporphyrin IX) group is bound covalently to the imidazole of His 87 (the "proximal histidine"). The wild-type beta chain is 146 residues long and heme is bound to it at His 92. Apohemoglobin is the heme-free analogue of hemoglobin; it exists predominantly as the $\alpha\beta$ -globin dimer.

Segments of polypeptide chains may be stabilized by folding into one of two common conformations, the alpha helix and the beta pleated sheet. In its native state, about 75% of the hemoglobin molecule is alpha-helical. Alpha-helical segments are separated by segments wherein the chain is less constrained. It is conventional to identify the alpha-helical segments of each chain by letters, e.g., the proximal histidine of the alpha chain is F8 (residue 8 of helix F). The non-helical segments are identified by letter pairs, indicating which helical segments they connect. Thus, nonhelical segment BC lies between helix B and helix C. In comparing two variants of a particular hemoglobin chain, it may be enlightening to attempt to align the helical segments when seeking to find structural homologies. For the amino acid sequence and

helical residue notation for normal human hemoglobin A₀ alpha and beta chains, see Bunn and Forget, supra, and Table 1 herein.

5 The tertiary structure of the hemoglobin molecule refers to the steric relationships of amino acid residues that are far apart in the linear sequence, while quaternary structure refers to the way in which the subunits (chains) are packed together. The tertiary and quaternary structure of the hemoglobin molecule have been discerned by X-ray
10 diffraction analysis of hemoglobin crystals, which allows one to calculate the three-dimensional positions of the very atoms of the molecule.

In its unoxxygenated ("deoxy", or "T" for "tense") form, the subunits of hemoglobin A (alpha1, alpha2, beta1, and beta2) form a tetrahedron having a twofold axis of
15 symmetry. The axis runs down a water-filled "central cavity". The subunits interact with one another by means of Van der Waals forces, hydrogen bonds and by ionic interactions (or "salt bridges"). The alpha1beta1 and
20 alpha2beta2 interfaces remain relatively fixed during oxygenation. In contrast, there is considerable flux at the alpha1beta2 (and alpha2beta1) interface. In its oxygenated ("oxy", or "R" for "relaxed" form), the intersubunit distances are increased.

25 The tertiary and quaternary structures of native oxyhemoglobin and deoxyhemoglobin are sufficiently well known that almost all of the nonhydrogen atoms can be positioned with an accuracy of 0.5 Å or better. For human deoxyhemoglobin, see Fermi, et al., J. Mol. Biol., 175:
30 159 (1984), and for human oxyhemoglobin, see Shaanan, J. Mol. Biol., 171: 31 (1983), both incorporated by reference.

Normal hemoglobin has cysteines at beta 93 (F9), beta 112 (G14), and alpha 104 (G11). The latter two positions are deeply buried in both the oxy and deoxy

states; they lie near the $\alpha_1\beta_1$ interface. Beta 93, however, in the oxy form is reactive with sulfhydryl reagents.

Native human hemoglobin has been fully reconstituted from separated heme-free alpha and beta globin and from hemin. Preferably, heme is first added to the alpha-globin subunit. The heme-bound alpha globin is then complexed to the heme-free beta subunit. Finally, heme is added to the half-filled globin dimer, and tetrameric hemoglobin is obtained. Yip, et al., PNAS (USA), 74: 64-68 (1977).

The human alpha and beta globin genes reside on chromosomes 16 and 11, respectively. Bunn and Forget, *infra* at 172. Both genes have been cloned and sequenced, Liebhaber, et al., PNAS 77: 7054-58 (1980) (alpha-globin genomic DNA); Marotta, et al., J. Biol. Chem., 252: 5040-53 (1977) (beta globin cDNA); Lawn, et al., Cell, 21:647 (1980) (beta globin genomic DNA).

Hemoglobin exhibits cooperative binding of oxygen by the four subunits of the hemoglobin molecule (two alpha-globins and two beta-globins in the case of Hgb A), and this cooperativity greatly facilitates efficient oxygen transport. Cooperativity, achieved by the so-called heme-heme interaction, allows hemoglobin to vary its affinity for oxygen. Hemoglobin reversibly binds up to four moles of oxygen per mole of Hgb.

Oxygen-carrying compounds are frequently compared by means of a device known as an oxygen dissociation curve. This curve is obtained when, for a given oxygen carrier, oxygen saturation or content is graphed against the partial pressure of oxygen. For Hgb, the percentage of saturation increases with partial pressure according to a sigmoid relationship. The P_{50} is the partial pressure at which the oxygen-carrying solution is half saturated with oxygen. It

is thus a measure of oxygen-binding affinity; the higher the P_{50} , the more loosely the oxygen is held.

When the oxygen dissociation curve of an oxygen-carrying solution is such that the P_{50} is less than that for whole blood, it is said to be "left-shifted."

The oxygen affinity of hemoglobin is lowered by the presence of 2,3-diphosphoglycerate (2,3-DPG), chloride ions and hydrogen ions. Respiring tissue releases carbon dioxide into the blood and lowers its pH (i.e. increases the hydrogen ion concentration), thereby causing oxygen to dissociate from hemoglobin and allowing it to diffuse into individual cells.

The ability of hemoglobin to alter its oxygen affinity, increasing the efficiency of oxygen transport around the body, is dependent on the presence of the metabolite 2,3- DPG. Inside the erythrocyte 2,3-DPG is present at a concentration nearly as great as that of hemoglobin itself. In the absence of 2,3-DPG "conventional" hemoglobin binds oxygen very tightly and would release little oxygen to respiring tissue.

Aging erythrocytes release small amounts of free hemoglobin into the blood plasma where it is rapidly bound by the scavenging protein haptoglobin. The hemoglobin-haptoglobin complex is removed from the blood and degraded by the spleen and liver.

Isolated alpha globin chains are monomers; exhibit high oxygen affinity but of course lack subunit cooperativity. Isolated beta globin chains aggregate to form a β_4 tetramer (HbH). The β_4 tetramer has a high but noncooperative oxygen affinity.

B. Blood Substitutes, Generally

It is not always practical to transfuse a patient with donated blood. In these situations, use of a red

blood cell substitute is desirable. The product must effectively transport O_2 , just as do red blood cells. ("Plasma expanders", such as dextran and albumin, do not transport oxygen.) The two types of substitutes that have been studied most extensively are hemoglobin solutions and fluorocarbon emulsions.

It is clear from the above considerations that free native hemoglobin A, injected directly into the bloodstream, would not support efficient oxygen transport about the body. The essential allosteric regulator 2,3-DPG is not present in sufficient concentration in the plasma to allow hemoglobin to release much oxygen at venous oxygen tension.

Nonetheless, solutions of conventional hemoglobin have been used as RBC substitutes. The classic method of preparing hemoglobin solutions employs outdated blood. The red cells are lysed and cellular debris is removed, leaving what is hopefully "stromal-free hemoglobin" (SFH).

Several basic problems have been observed with this approach. The solution must be freed of any toxic components of the red cell membrane without resorting to cumbersome and tedious procedures which would discourage large-scale production. DeVenuto, "Appraisal of Hemoglobin Solution as a Blood Substitute", Surgery, Gynecology and Obstetrics, 149: 417-436 (1979).

Second, as expected, such solutions are "left-shifted" (lower P_{50}) as compared to whole blood. Gould, et al., "The Development of Polymerized Pyridoxylated Hemoglobin Solution as a Red Cell Substitute", Ann. Emerg. Med. 15: 1416- 1419 (Dec. 3, 1986). As a result, the oxygen affinity is too high to unload enough oxygen into the tissues. Benesch and Benesch, Biochem. Biophys. Res. Comm., 26:162-167 (1967).

Third, SFH has only a limited half-life in the circulatory system. This is because oxy Hgb partially dissociates into a dimer ($\alpha\beta$) that is rapidly cleared from the blood by glomerular filtration and binding to circulating haptoglobin. If large amounts of soluble hemoglobin are introduced into the circulation, glomerular filtration of the dimers may lead to a protein and iron load on the kidney capable of causing renal damage. Bunn, H.F., et al. (1969) The renal handling of hemoglobin I. Glomerular filtration. J. Exp. Med. 129:909-923; Bunn, H.F., and J.H. Jandl; (1969) The renal handling of hemoglobin II. Catabolism. J. Exp. Med. 129:925-934; Lee, R.L., et al. (1989) Ultrapure, stroma-free, polymerized bovine hemoglobin solution: Evaluation of renal toxicity (blood substitutes) J. Surgical Res. 47:407-411; Feola, M., et al. (1990) Nephrotoxicity of hemoglobin solutions. Biomat. Art. Cell Art. Org., 18(2):233-249; Tam, S.C. and J. T.F. Wong (1988) Impairment of renal function by stroma-free hemoglobin in rats. J. Lab. Clin. Med. 111:189-193.

Finally, SFH has a high colloid osmotic pressure (COD). Thus, administration of SFH in a dose that would have the same oxygen-carrying capacity as a unit of packed red blood cells is inadvisable, since the high osmotic pressure (60mm Hg) would cause a massive influx of water from the cells into the bloodstream, thus dehydrating the patient's tissues. This consideration limits the dose of SFH to that which provide a final concentration of about 6-8 gm Hgb/dl.

In an effort to restore the desired P_{50} , researchers added 2,3-DPG to the hemoglobin solution. Unfortunately, 2,3-DPG was rapidly eliminated from the circulation. Scientists then turned to other organic phosphates, particularly pyridoxal phosphate. Like 2,3-DPG, these compounds stabilized the "T state" of the Hgb by

forming a salt bridge between the N-termini of the two beta chains. The pyridoxylated hemoglobin had a P_{50} of 20-22 torr, as compared to 10 torr for SFH and 28 torr for whole blood. While this is an improvement over SFH, the pyridoxylated Hgb remains "high affinity" relative to whole blood.

C. Naturally Occurring Cysteine Substitution Mutants of Hemoglobin (Non-Polymerizing)

There are a few known naturally occurring mutants of human hemoglobin in which a cysteine residue is substituted for another residue of normal hemoglobin Ao.

In hemoglobin Nigeria, the mutation is α 81 Ser \rightarrow Cys; no disulfide is formed. Haris, et al., Blood, 55(1):131-137 (1980). In Hemoglobin Rainier, an intrasubunit disulfide is formed between the wild type F9(93) β Cysteine and the cysteine introduced by replacement of the Tyr at HC2(145) β . Greer, et al., Nature [New Biology], 230:261-264 (1971). Hemoglobin Nunobiki (β 141 Drg \rightarrow Cys) also features a non-polymerizing cysteine substitution. In both Hb Rainier and Hb Nunobiki, the new cysteine residues are on the surface of the tetramer.

D. Naturally Occurring Polymerizing or Polymeric Hemoglobins

Three other human mutants are known which polymerize as a result of formation of intermolecular (first tetramer to second tetramer) disulfide bridges. In Hemoglobin Porto Alegre, the Ser at A6(9) β is replaced by Cysteine, and since this cysteinyl residue is externally oriented, spontaneous polymerization occurs, and results in formation of a dodecamer with three Porto Alegre tetramers

linked by disulfide bonds. An octamer has also been made by a 1:1 mixture of Porto Alegre hemoglobin and normal hemoglobin. Tondo, Biochem. Biophys. Acta, 342:15-20 (1974); Tondo, An. Acad. Bras. Cr., 59:243-251 (1987).

5 Hb Mississippi is characterized by a cysteine substitution in place of Ser CD3(44) β . Hemolysates of a patient were subjected to gel filtration column chromatography, and 48.8% eluted in the void volume. Since the molecular weight exclusion was about 600kD, this
10 suggested that Hb MS polymers are composed of ten or more hemoglobin tetramers. Adams, et al., Hemoglobin, 11(5):435-452 (1987).

A β 83(EF7)Gly \rightarrow Cys mutation characterizes Hemoglobin Ta Li. This mutant showed slow mobility in
15 starch gel electrophoresis, indicating that it was a polymer.

Polymeric mouse hemoglobins have been reported. In BALB/cJ mice, there is a reactive cysteinyl residue near the NH₂-terminal of the beta chain (β -13 in the mouse).
20 This mouse mutant has been compared to Hemoglobin Porto Alegre, which likewise has a cysteinyl residue in the A-helix of the beta chain. Octamer formation is most common. However, each tetramer has two extra cysteinyl residues, one on each β -chain, that may react with different
25 tetramers; "this explains why aggregates larger than octamers occur". Benaventura and Riggs, Science, 149:800-802 (1967); Riggs, Science, 147:621-623 (1965).

Macaques also exhibit a polymerizing hemoglobin variant. Takenaka, et al., Biopchem. Biophys. Acta,
30 492:433-444 (1977); Ishimoto, et al., J. Anthropol. Soc. Nippon, 83(3):233-243 (1975). This mutant has been compared to the Ta Li variant in humans.

Both amphibians and reptiles possess polymerizing hemoglobins. For example, in the bullfrog, hemoglobin

"Component C" polymerizes by disulfide bond formation between tetramers. This is said to be primarily dependent on cysteinyl residues of the alpha chain. Tam, et al., J. Biol. Chem., 261:8290-94 (1986).

5 The extracellular hemoglobin of the earthworm (Lumbricus terrestris) has a complex structure. There are twelve subunits, each being a dimer of structure (abcd)₂, where "a", "b", "c", and "d" denote the major heme containing chains. The "a", "b", and "c" chains form a
10 disulfide-linked trimer. The whole molecule is composed of 192 heme-containing chains and 12 non-heme chains, and has a molecular weight of 3800 kDa. Other invertebrate hemoglobins are also large multi-subunit proteins.

 The brine shrimp Artemia produces three polymeric
15 hemoglobins with nine genetically fused globin subunits. Manning, et al., Nature, 348:653 (1990). These are formed by variable association of two different subunit types, a and b β . Of the eight intersubunit linkers, six are 12 residues long, one is 11 residues and one is 14 residues.

20 *E. Artificially Crosslinked Hemoglobins (Non-Polymerizing)*

 The properties of hemoglobin have been altered by specifically chemically crosslinking the alpha chains between the Lys99 of alpha1 and the Lys99 of alpha2. Walder, U.S. 4,600,531 and 4,598,064; Snyder, et al., PNAS
25 (USA) 84: 7280-84 (1987); Chatterjee, et al., J. Biol. Chem., 261: 9927-37 (1986). The beta chains have also been chemically crosslinked. Kavanaugh, et al., Biochemistry, 27: 1804-8 (1988). Kavanaugh notes that the beta N-termini are 16 Å apart in the T state and 20 Å apart in the
30 R state. Not surprisingly, the introduction of a DIDS bridge between the N-termini of T state hemoglobin hindered the shift to the R state, thereby decreasing the

O₂ affinity of the molecule. While the Kavanaugh analogue has desirable oxygen binding and renal clearance characteristics, it too is obtained in low yield.

Hoffman and Nagai, USP 5,028,588 suggest that the T state of hemoglobin may be stabilized by intersubunit (but intratetrameric) disulfide crosslinks resulting from substitution of cysteine residues for other residues. A particularly preferred crosslink was one connecting beta Gly Cys with either alpha G17 (Ala→Cys) or G18 (Ala→Cys).

F. Artificially Crosslinked Hemoglobin (Polymerizing)

Bonsen, USP 4,001,401, USP 4,001,200, and USP 4,053,590 all relate to polymerization of red blood cell-derived hemoglobin by chemical crosslinking. The crosslinking is achieved with the aid of bifunctional or polyfunctional crosslinking agents, especially those reactive with exposed amino groups of the globin chains. The result of the crosslinking reaction is a polydisperse composition of covalently cross-linked aggregates.

Bonhard, USP 4,336,248 discloses chemical crosslinking of hemoglobin molecules to each other, or to serum proteins such as albumin.

Bonhard, USP 4,777,244 sought to stabilize the dialdehyde-cross-linked hemoglobins of the prior art, which tended to polymerized further while in storage, by adding a reducing agent to stabilize the azomethine bond.

Bucci, USP 4,584,130, at col. 2, comments that "the polyhemoglobin reaction products are a heterogeneous mixture of various molecular species which differ in size and shape. The molecular weights thereof range from 64,500 to 600,000 Daltons. The separation of individual molecular species from the heterogeneous mixture is virtually impossible. In addition, although longer retention times

in vivo are obtained using polyhemoglobins, the oxygen affinity thereof is higher than that of stroma-free hemoglobin."

According to Tye, USP 4,529,179, "most workers have chosen to form the random intermolecular crosslinked polymers of hemoglobin because they believed that the 65,000 Dalton tetramer was filtered by the glomerulus.... Usually the amino groups of lysine on the surface of the hemoglobin molecule are coupled with a bifunctional reactant such as gluteraldehyde or suberimide. There are 42 lysines available for reaction per hemoglobin tetramer so that one can get an infinite number of different inter [or] intra molecular crosslinks making various polymers of hemoglobin.... The random polymerization is difficult to control and gives a range between two and ten tetramers per polymer.... No one has yet standardized an analytical scheme to establish lot to lot variability of structure and function.... [Polymerized pyridoxylated hemoglobin has] a profound chemical heterogeneity making it difficult to study as a pharmaceutical agent."

G. Fused Genes and Proteins, Generally

Genes may be fused together by removing the stop codon of the first gene, and joining it in phase to the second gene. Parts of genes may also be fused, and spacer DNAs which maintain phase may be interposed between the fused sequences. The product of a fused gene is a single polypeptide, not a plurality of polypeptides as is expressed by a polycistronic operon. Different genes have been fused together for a variety of purposes. Thus, Gilbert, U.S. 4,338,397 inserted a rat preproinsulin gene behind a fragment of the E. coli penicillinase gene. His purpose was to direct E. coli transformants to secrete the

expression product of the fused gene. Fused genes have also been prepared so that a non- antigenic polypeptide may be expressed already conjugated to an immunogenic carrier protein.

5 The use of linker DNA sequences to join two different DNA sequences is known. These linkers are used to provide restriction sites for DNA cleavage, or to encode peptides having a unique character that facilitates purification of the encoded fusion protein or a fragment thereof. See, e.g., Rutter, U.S. 4,769,326.

10 Hallewell, et al., J. Biol. Chem., 264: 5260-68 (1989) prepared an analogue of CuZn superoxide dismutase. Each dismutase molecule is a dimer of two identical subunits; a copper ion and a zinc ion are liganded to the subunit. The dimer interaction in CuZn superoxide dismutase is so strong that the subunits have not been separated without inactivating the enzyme. The enzyme has considerable conformational similarity to immunoglobulins; Hallewell, et al., joined two human superoxide dismutase genes, either directly or with DNA encoding a 19-residue human immunoglobulin IgA1 hinge region and expressed the fused genes in a transformed host. In attempting to express the directly joined genes, recombination occurred to eliminate one of the tandem genes in some plasmid molecules. Hallewell, et al., postulated that the direct connection distorted the dimer, causing the exposure of hydrophobic areas which then had a toxic effect. This would have provided selection pressure favoring gene deletion. No recombination was detected with the IgA1 linker construction.

30 Hoffman, et al., WO88/09179 describe the production, in bacteria and yeast, of hemoglobin and analogues thereof. The disclosed analogues including hemoglobin proteins in which one of the component

polypeptide chains consists of two alpha or two beta globin amino acid sequences covalently connected by peptide bonds, preferably through an intermediate linker of one or more amino acids, without branching. In normal hemoglobin, the alpha and beta globin subunits are non-covalently bound.

SUMMARY OF THE INVENTION

The present invention relates to multimeric hemoglobin-like proteins wherein two or more tetramers or pseudotetramers are covalently bonded. Between any pair of covalently linked tetramers, the covalent linkage may take the form of a crosslink between two cysteine residues of different polypeptide chains, or of a peptide linker connecting the "carboxy most" residue of a globin-like domain of one tetramer with the "amino most" residue of a similar domain of a second tetramer.

Preferably, the multimeric hemoglobin-like protein-containing composition is at least 50% monodisperse, more preferably, at least 95% monodisperse.

Although free hemoglobin purified from natural sources may be polymerized by chemical crosslinking to increase halflife via increased molecular weight, and to reduce oncotic pressure, all such preparations are heterogeneous. Monodispersability can be achieved only by laborious purification.

The present invention provides means of exerting strict control over the degree of polymerization of hemoglobin tetramers. The ability to strictly control formation of multimers will greatly facilitate purification and characterization of the final product and will reduce the chance of adverse reaction to minor components. It is also believed that a more monodisperse composition will have greater consistency of clinical effect.

Hemoglobin also may be made by expression of alpha and beta globin genes in the same or different host cells, and subsequent assembly of the expressed alpha and beta globins, with heme, to form hemoglobin. While the introduction of suitable Cys codon mutations into the globin genes facilitates the production of a crosslinked multimeric hemoglobin, the expression product in general, will not be essentially monodisperse. Hemoglobin is composed of two alpha and two beta globin subunits. Both alpha globin subunits are natively expressed from a single alpha globin gene, and both beta globin subunits, from a single beta globin gene. Thus, if an alpha globin gene is expressed which contains a single Cys codon substitution, the assembled tetramer will contain two alpha globin subunits, each with a crosslinkable Cys. One Cys could crosslink to a second tetramer, and the other to a third, thus resulting in formation of a higher order oligomer.

In one embodiment, the multimeric protein is an octamer consisting essentially of two tetramers which are covalently crosslinked. To avoid unwanted polymerization, each tetramer has only a single participating cysteinyl residue, whose thiol groups are reacted either with each other (under oxidizing conditions, forming a disulfide bond) or with a thiol-reactive crosslinking agent, to form the crosslink.

A fused gene which encodes a single polypeptide comprising two globin-like domains may be mutated so as to provide an externally crosslinkable Cys in only one of the two otherwise substantially identical domains of the resulting pseudodimeric polypeptides. This pseudodimer may then be assembled with the complementary subunits to form a tetramer with only the single cysteine. Two such tetramers, finally, may be crosslinked to obtain the octamer, preferably in essentially monodisperse form.

If the formation of a higher order multimer, such as a dodecamer, is desired, the component pseudotetramers, each having a single externally crosslinkable cysteine, are each covalently attached to a reactive site of a polyfunctional crosslinker having a suitable half-life in the bloodstream.

Another way of obtaining a multimeric hemoglobin instead of crosslinking two or more pseudotetramers, to combine their pseudodimeric subunits into a single pseudooligomer that is shared by all of the component pseudotetramers of the multimeric hemoglobin. For example a pseudooctameric polypeptide, comprising eight alpha globin-like domains, joined covalently by peptide bonds (typically with a peptide spacer), may be assembled with eight individual beta globin-like subunits to form a tetra-tetrameric human hemoglobin-like protein. Higher order multimers may be prepared simply by expressing a suitable pseudooligomer and assembling it with the complementary monomeric subunits.

The preparation of multimeric hemoglobins with a genetically fused pseudooligomeric backbone avoids the disadvantages of chemical crosslinking. The latter is inefficient and often requires deoxygenation of the hemoglobin solution and the presence of another molecule (e.g., inositol hexaphosphate or 2,3-DPG) to prevent competing reactions.

In the embodiments discussed above, an essentially monodisperse multimeric hemoglobin is achieved by limiting the number of externally crosslinkable cysteines to one per tetramer. However, it is possible to have more than one externally crosslinkable cysteine per tetramer, provided that they are so positioned that after one is crosslinked to a foreign tetramer, other foreign

tetramers are sterically prevented from crosslinking to the remaining cysteines of the original tetramer.

The multimeric proteins of the present invention, particularly at higher levels of polymerization, may prolong the half-life of recombinant hemoglobin by reducing extravasation and glomerular filtration of dissociated subunits in vivo compared to native human hemoglobin. Studies of halflife as a function of macromolecular size indicate a correlation between increased size and increased circulatory halflife for chemically crosslinked Hb as well as other macromolecules. Preferably, in humans, the half-life exceeds 9 hours at a dose of at least 1 gm/kgm body weight. This would be expected to correspond to a half-life of about 3 hours in rats given a comparable dose.

Intravascular retention may also be enhanced by engineering the tetramer crosslinking sites so that the haptoglobin binding sites of the tetramers are wholly or partially occluded. Independent mutations may also be made to sterically hinder haptoglobin binding, or to electrostatically repel or sterically hinder the approach of agents which otherwise might degrade the crosslink.

The multimeric proteins of the present invention may also increase oncotic pressure because the number of oxygen binding heme groups per polytetramer of order "n" is "n" times the number per tetramer. Independent of size, the oncotic pressure for a given concentration of heme groups in a solution of polytetrameric Hb is expected to be (1/n) times that of an equimolar solution of heme contained in tetrameric Hb. Because of oncotic pressure effects, the maximum concentration of free tetrameric Hb that may be introduced into the blood stream is less on a per volume basis than the concentration of Hb normally carried in intact red blood cells. Reduction of oncotic pressure is

therefore useful in increasing the per volume oxygen carrying capacity of a blood substitute.

In a preferred embodiment, one or more globin-like domains contain mutations which reduce the oxygen-binding affinity of the hemoglobin analogue in solution so as to approach the oxygen-binding characteristics of whole blood.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Plasmid pSGE1.1E4. This plasmid bears a polycistronic operon which comprises the pTAC promoter and genes encoding a di-alpha globin and a beta globin. It also carries tetracycline and ampicillin resistance genes, and the lacI gene.

Figure 2 Shows the sequence [SEQ ID NO:1] of a preferred synthetic gene for expression of (des-Val)-alpha-(Gly)-alpha and des-Val beta globin. This gene is carried by pSGE1.1E4. A shows the region (EcoRI to PstI) containing Shine-Delgarno ribosomal binding sites (SD#1 and SD#2), the sequence expressing the octapeptide (Met...Glu) (SEQ ID NO:25) which serves as a cotranslational coupler, and the sequence encoding the two nearly identical alpha globin-like polypeptides and the interposed Gly-Gly linker. The first alpha globin sequence begins "Met-Leu", that is, it contains an artifactual methionine, omits the valine which is the normal first residue of mature alpha globin, and continues with the second residue, leucine. The residues are numbered 1 to 141 (SEQ ID NO:26). The second alpha globin sequence begins "Val-Leu", immediately after the underlined "Gly-Gly" linker. The residues are numbered 1' to 141' (SEQ ID NO:27). Start and stop codons are underlined. B shows the analogous region (PstI to HindIII) containing the coding sequence for des-Val beta globin.

The beta residues are numbered 1 to 146 (SEQ ID NO:28). A and B are connected at the PstI site to form a single polycistronic operon.

5 When a three letter amino acid code is singly underlined, this indicates that the residue is a potential site for an Xaa → Cys mutation to provide a crosslinkable site. The mutations should be made asymmetrically, i.e., only one region of a di-alpha or di-beta gene, so only one crosslink is added per tetramer. While, in Figure 2, the
10 sites are marked only on the first copy of the alpha gene, they could instead be in the second copy. For convenience, the appropriate beta globin mutation sites are also marked. However, these mutations should be made in only one beta-globin of a di-beta globin gene.

15 Doubly underlined amino acid codes identify sites where formation of two disulfide bonds (or per subunit) would be sterically hindered, so use of a di-alpha or di-beta construction is unnecessary.

20 Residues which are candidate sites for mutations to block haptoglobin binding are boxed.

Figure 3 is a stylized representation of one form of pseudooctameric Hgb, in which the octameric hemoglobin is formed by linking or crosslinking two molecules of an asymmetric di-alpha Hgb.

25 Figure 4 depicts coiled coil crosslinkers suitable for joining (a) four or (b) six Hgb tetramers. Fig. 4(c) is a top view of a 4-helical bundle, with attachment sites marked.

30 Figure 5 Schematic showing how cysteine mutations can favor formation of octamer without genetic fusion of subunits.

Figure 6 Proposed α_1 - β_2 globin pseudodimer.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

A hemoglobin is a protein which contains heme (ferroprotoporphyrin IX) and that binds oxygen at a respiratory surface (skin, gills, trachea, lung, etc.) and transports the oxygen to inner tissues, where it is released and used for metabolism. In nature, low molecular weight hemoglobins (16-120 kilodaltons) tend to be enclosed in circulating red blood cells while the larger polymeric hemoglobins circulate freely in the blood of hemolymph.

For the purpose of the appended claims, a hemoglobin-like protein is a protein with the following characteristics:

- (a) it is sufficiently soluble in blood to be clinically useful as a blood substitute;
- (b) it reversibly binds oxygen, under physiological conditions;
- (c) each polypeptide chain comprises at least one globin-like domain (as defined below); and
- (d) each globin-like domain bears (or is capable of incorporating) a heme prosthetic group;

A multimeric hemoglobin-like protein is further characterized as follows:

- (e) it is composed of two or more polypeptide chains;
- (f) it is composed of two or more tetramers, each tetramer comprising four globin-like domains, and

(g) each component tetramer is covalently attached to at least one other component tetramer.

5 Preferably, the hemoglobin-like proteins of the present invention have a P_{50} of 2 to 45 torr, more preferably 24 to 32 torr, at 37°C, in blood. Preferably, they also exhibit some degree of cooperativity. Also, they desirably have an intravascular retention at least comparable to that of normal human hemoglobin administered
10 as a blood substitute.

Tetrameric hemoglobin-like proteins have four globin-like domains, octameric hemoglobin-like proteins have eight globin-like domains, and so forth. The term "multimeric" covers any hemoglobin-like protein comprising
15 (4 x n) globin-like domains, where $n > 1$.

A pseudomeric hemoglobin-like protein is one for which the number of globin-like domains is greater than the number of component polypeptide chains, i.e., at least one chain comprises at least two globin-like domains. The
20 pseudoheterotetrameric hemoglobin-like proteins, for example, may be composed of (a) one di-alpha globin-like and two beta globin-like polypeptides, (b) two alpha globin-like and one di-beta globin-like polypeptides, (c) one di-alpha globin-like and one di-beta globin-like
25 polypeptides, (d) one fused alpha/beta globin-like polypeptide and separate alpha and beta globin-like polypeptides, or (e) two fused alpha/beta globin-like polypeptides. The term "tetramer" includes "pseudotetramers."

30 A "genetically fused hemoglobin" is a hemoglobin-like protein comprising at least one "genetically fused globin-like polypeptide", (globin pseudooligomer), the latter comprising two or more globin-like domains which may

be the same or different and which are connected directly, or through an amino acid or peptide linker. A di-alpha globin-like polypeptide is one which consists essentially of two alpha-globin-like polypeptide sequences (domains) connected by peptide bonds between the normal C- terminus of the first alpha-globin-like polypeptide (domain) and the normal N-terminus of the second alpha-globin-like polypeptide (domain). These two sequences may be directly connected, or connected through a peptide linker of one or more amino acids; the term "peptide bonds" is intended to embrace both possibilities. Alpha globin chains crosslinked at the N- and C-terminals other than by peptide bonds (e.g., by DIDS) are not di-alpha globins. The di-alpha globin-like polypeptide must be capable of folding together with beta globin and incorporating heme to form functional hemoglobin-like protein. The di-beta globin-like polypeptide is analogously defined. A di-alpha or di-beta globin-like polypeptide with a mutation in only one of the component domains is called "asymmetric".

It is also possible to provide an "alpha/beta-globin-like pseudodimer" in which an alpha globin-like sequence is connected by peptide bonds to a beta globin-like sequence. This "alpha/beta globin-like polypeptide", and the di-alpha and di-beta globin-like polypeptides, may collectively be referred to as "pseudodimeric globin-like polypeptides" or as "diglobins". By extension, a hemoglobin-like protein comprising a di-alpha, a di-beta, or a alpha/beta globin-like polypeptide is a "pseudotetramer".

Pseudotetramers which bear only a single externally crosslinkable cysteine may be referred, by way of shorthand, as "mono-cys" molecules. However, the use of this term should not be taken as implying that the tetramer may not comprise other cysteines. A "mono-cys"

pseudotetramer is merely one which has only a single cysteine which can participate to a significant degree in crosslinking reactions with a cysteine residue of a second pseudotetramer.

5 A hemoglobin-like protein is said to be heteromeric if at least two of its globin-like domains are different. Since conventional human hemoglobin is composed of two alpha globins and two beta globins, it is a heterotetramer. A multimeric human hemoglobin-like protein
10 is a heteromer wherein each tetramer or pseudotetramer has two human alpha globin-like domains and two human beta globin-like domains.

The Globin-Like Domain

15 The globin-like domains may be, but need not be, one per polypeptide chain, and they need not correspond exactly in sequence to the alpha and beta globins of normal human hemoglobin. Rather, mutations may be introduced to alter the oxygen affinity (or its cooperativity, or its
20 dependence on pH, salt, temperature, or other environmental parameters) or stability (to heat, acid, alkali, or other denaturing agents) of the hemoglobin, to facilitate genetic fusion or crosslinking, or to increase the ease of expression and assembly of the individual chains. Guidance
25 as to certain types of mutations is provided, e.g., by Hoffman and Nagai, U.S. Patent 5,028,588, and Ser. No. 07/443,950, incorporated by reference herein. The present invention further includes molecules which depart from those taught herein by gratuitous mutations which do not substantially affect biological activity.

30 A "globin-like domain" is a polypeptide domain which is substantially homologous with a globin subunit of a naturally occurring hemoglobin. A "vertebrate,"

"mammalian" or "human" globin-like domain is one which is substantially homologous with a globin subunit of, respectively, a naturally occurring vertebrate, mammalian or human hemoglobin.

5 A human alpha globin-like domain or polypeptide is native human alpha globin or a mutant thereof differing from the native sequence by one or more substitutions, deletions or insertions, while remaining substantially homologous (as hereafter defined) with human alpha globin,
10 and still capable of incorporating heme and associating with beta globin. The term "human alpha globin-like domain" is intended to include but not be limited to naturally occurring human alpha globins, including normal human alpha globin. A beta globin-like domain or
15 polypeptide is analogously defined. Subunits of animal hemoglobins or mutants thereof which are sufficiently homologous with human alpha or beta globin are embraced by the term "human alpha or beta globin-like domain or polypeptide." For example, the subunits of bovine
20 hemoglobin are within the scope of these terms.

 In determining whether a polypeptide is substantially homologous to alpha (or beta) globin, sequence similarity is an important but not exclusive criterion. Sequence similarity may be determined by
25 conventional algorithms, which typically allow introduction of a small number of gaps in order to achieve the best fit. A human alpha-globin-like domain will typically have at least about 75% sequence identity with wild-type human alpha globin, and greater homology with human alpha globin
30 than with human beta globin. However, a polypeptide of lesser sequence identity may still be considered "substantially homologous" with alpha globin if it has a greater sequence identity than would be expected from chance and also has the characteristic higher structure

(e.g., the "myoglobin fold") of alpha globin, the ability to incorporate heme, and oxygen-binding activity. (Note that, as elsewhere explained, an alteration in oxygen affinity (P50), intravascular retention, or cooperativity may be desired, and does not render the mutant nonhomologous if it can still contribute to reversible oxygen-binding activity.) By way of comparison, Artemia's heme-binding domains are considered homologous with myoglobin even though the primary sequence similarity is no more than 27%, as alignment of the heme-binding domains around their conserved residues and the residues conserved in other hemoglobins (i.e., involved in heme contacts or in determining the relationship of the helical segments to each other) suggested that the Artemia domains possessed the classical globin helices A to H with their corresponding turns, as well as various conserved globin family residues. Also, among the serine protease inhibitors, there are families of proteins recognized to be homologous in which there are pairs of members with as little as 30% sequence homology.

Over a hundred mutants of human hemoglobin are known, affecting both the alpha and beta chains, and the effect of many of these mutations on oxygen-binding and other characteristics of hemoglobin are known. The human alpha and beta globins themselves differ at 84 positions. In addition, interspecies variations in globin sequence have been extensively studied. Dickerson, Hemoglobin: Structure, Function, Evolution and Pathology, ch. 3 (1983) reported that in 1982, the 60 known vertebrate alpha globins had identical residues at 23 of their 141 positions, while for the 66 vertebrate beta globins considered, 20 of the 146 amino acids are identical. The 60 vertebrate myoglobins, which also belong to the globin family, had 27 invariant amino acids out of 153 positions.

If only mammals are considered, then the invariant amino acids are 50/141 for the alpha globins, 51/146 for the beta globins, and 71/153 for the myoglobins. Invariant positions cluster around the centers of activity of the molecule: the heme crevice and the intersubunit contacts. Of the variable amino acids, some diverge from the consensus sequence for only a small fraction of the species considered.

The number of total differences between human alpha globin and selected other vertebrate alpha globins is as follows: rhesus monkey (4), cow (17), platypus (39), chicken (35), human zeta (embryonic) (61), carp (71), and shark (88). For invertebrate globins the divergences are sea lamprey (113), mollusc (124), Glycera (marine bloodworm) (124) and Chironomus (midge) (131). Turning to the beta globin family, the differences of human beta globin from other vertebrate beta globins are rhesus monkey (8), human delta globin (10), cow beta globin (25), cow gamma globin (33), human gamma globin (39), human epsilon (embryonic) globin (36), platypus (34), chicken (45), shark (96), sea lamprey (123), mollusc (127), Glycera (125) and Chironomus (128).

Many of these differences may be misleading -- variable amino acids may exhibit only "conservative substitutions" of one amino acid for another, functionally equivalent one. A "conservative substitution" is a substitution which does not abolish the ability of a globin-like polypeptide (or domain) to incorporate heme and to associate with alpha and beta globin subunits to form a tetrameric (or pseudotetrameric) hemoglobin-like protein which, in keeping with the definition thereof, will reversibly bind oxygen. The following resources may be used to identify conservative substitutions (and deletions or insertions):

(a) data on functional hemoglobin mutants (over a hundred such mutants exist);

(b) data on sequence variations among vertebrate, especially mammalian, alpha globins and beta globins;

(c) data on sequence variations among vertebrate, especially mammalian, myoglobins;

(d) data on sequence variations between vertebrate and invertebrate globins, or among the invertebrate globins;

(e) data on the three-dimensional structures of human hemoglobin and other oxygen-binding proteins, and molecular modelling software for predicting the effect of sequence changes on such structures; and

(f) data on the frequencies of amino acid changes between members of families of homologous proteins (not limited to the globin family). See, e.g., Table 1-2 of Schulz and Schirmer, Principles of Protein Structure (Springer-Verlag: 1979) and Figure 3-9 of Creighton, Proteins: Structure and Molecular Properties (W.H. Freeman: 1983).

While the data from (a) - (d) is most useful in determining tolerable mutations at the site of variation in the cognate proteins, it may also be helpful in identifying tolerable mutations at analogous sites elsewhere in the molecule. Based on the data in category (f), the following exchange groups may be identified, within which substitutions of amino acids are frequently conservative:

I small aliphatic, nonpolar or slightly polar residues -

Ala, Ser, Thr (Pro, Gly)

II negatively charged residues and their amides - Asn, Asp, Glu, Gln

III positively charged residues -

His, Arg, Lys

IV large aliphatic nonpolar residues -

Met, Leu, Ile, Val (Cys)

V large aromatic residues -

Phe, Tyr, Trp

5

Three residues are parenthesized because of their special roles in protein architecture. Gly is the only residue without a side chain and therefore imparts flexibility to the chain. Pro has an unusual geometry which tightly constrains the chain. Cys can participate in disulfide bonds which hold proteins into a particular folding. Note that Schulz and Schimer would merge I and II above. Note also that Tyr, because of its hydrogen bonding potential, has some kinship with Ser, Thr, etc.

10

15

In general, functionality is less likely to be affected by mutations at surface residues, at least those not involved in either the heme crevice or the subunit contacts. In addition, "loops" connecting alpha helices, as well as free amino or carboxy termini, are more tolerant of deletions and insertions.

20

A "Met FX alpha globin" is an alpha globin-like polypeptide comprising an N-terminal methionine, a oligopeptide which acts as a recognition site for Factor Xa (e.g., Ile-Glu- Gly-Arg) (SEQ ID NO:2), and an alpha globin-like sequence (e.g., Val-His-Leu- Thr-Pro...) (SEQ ID NO:3) which may correspond to wild-type alpha globin or to a mutant thereof as taught herein. The term "Met FX alpha globin" is some-times abbreviated as "FX alpha globin". "FX beta globin" is an analogously defined beta globin-like polypeptide.

25

30

"Met-alpha globin" is an alpha globin-like polypeptide with an extra N-terminal methionine. The second amino acid is valine, which is the first amino acid of mature wild-type alpha globin. Met-beta globin is

analogously defined. A "Des-FX alpha globin" gene (or "dFX alpha globin") is a Met-alpha globin gene obtained by excising the FX codons from a Met-FX alpha globin gene. Note that "Met-Hgb" is used to refer to methionyl Hgb formed from methionyl-alpha globin and methionyl-beta globin.

"Des-Val-alpha globin" (or "dVal alpha globin") is an alpha globin-like polypeptide wherein methionine is substituted for the valine which begins the sequence of mature wild-type alpha globin. Des-Val-beta globin is analogously defined. Des-Val-alpha/alpha globin (di-Des-Val-alpha globin) is a "di- alpha globin" in which a "Des-Val-alpha" sequence is linked via an appropriate peptidyl linker to an alpha globin-like sequence which begins with Val.

Low Affinity Mutants

The term "low affinity hemoglobin-like protein" refers to a hemoglobin-like protein having a P_{50} which is at least 10% greater than the P_{50} of cell free normal hemoglobin A_0 under the same conditions. Preferably, the protein, if used as a blood substitute, qualifies as a low affinity protein, and more preferably, its P_{50} is closer to the P_{50} of whole blood cells than to that of cell free hemoglobin.

Low affinity mutant hemoglobins, i.e., those with "right shifted" oxygen equilibrium binding curves relative to cell-free normal hemoglobin, have many potential uses. Most notably, mutant hemoglobins that have an oxygen affinity similar to whole red blood cells may be used as an oxygen- carrying transfusion substitute in place of donated red blood cells, eliminating the risk of infection and alleviating problems with supply. Cell-free native human hemoglobin cannot function as a transfusion substitute,

among other reasons because oxygen is bound too tightly. In addition, because cell-free hemoglobin solutions do not need to be cross-matched and are expected to have a longer shelf life than whole blood, low affinity hemoglobin solutions may be widely used in situations where whole blood transfusion is not feasible, for example in an ambulance or on a battlefield. Mutant hemoglobins that have an even lower oxygen affinity than red blood cells may in fact delivery oxygen more effectively in many situations. Mutant hemoglobins that have a somewhat higher oxygen affinity than whole blood (but a lower affinity than cell-free native human hemoglobin) will still function as an adequate transfusion substitute and may in fact deliver oxygen more effectively than red blood cells in some situations. This is because oxygen is released directly to plasma form hemoglobin-based solutions, without the need to diffuse through the red cell membrane, and because cell-free hemoglobin may penetrate into regions not accessible to red blood cells. As an example, low affinity mutant hemoglobin is expected to deliver oxygen effectively during coronary artery balloon angioplasty procedures, whereas circulation of red blood cells is obstructed during such procedures. Low affinity mutant hemoglobin may also be useful as a perfusion component in organ preservation prior to transplantation or as a mammalian cell culture additive.

Possible low affinity mutants are discussed in detail, by way of example and not of limitation, in Table 1 (natural low affinity hemoglobin mutants) and Table 2 (candidate non-naturally occurring low affinity hemoglobin mutants) of Hoffman, et al., U.S. Patent 5,028,588. Low affinity mutants of particular interest are the Presbyterian (beta Lys¹⁰⁸) beta Phe⁶³, beta Ile⁶⁷, and Kansas (beta Thr¹⁰²) mutants.

An unexpected and surprising change in oxygen binding characteristics of hemoglobin was observed upon replacement of the N-terminal valine with methionine. Hemoglobin A₀ purified from blood has a P₅₀ value of 4.03 with N=2.8 when measured at 25°C. DesFX-hgb produced in E. coli, a hemoglobin identical to A₀ except for the addition of a methionine at the N-termini of the alpha and beta chains, has essentially the same P₅₀ and N values. Thus, the addition of a methionine, without altering the adjacent valine residue, has little or no effect on oxygen binding. On the other hand, a higher P₅₀ value, 6.6, was observed for desVal-hgb produced in E. coli, a hemoglobin in which the normal N-terminal valine of each chain was replaced with methionine. Cooperativity, as measured by N, was virtually the same, however, for all three molecules.

A similar comparison was made for two hemoglobins each containing the Presbyterian mutation, one produced in E. coli and one in yeast. The E. coli hemoglobin was constructed with a Des-Val alpha chain, i.e., the N-terminus had the normal valine replaced with methionine. Oxygen binding was characterized by P₅₀=19.8, N=2.5 at 25°C. and by P₅₀=34.5 and N=2.5 at 37°C. The corresponding yeast coding region begins with an additional methionine codon in front of the normal valine codon. Because this initial methionine is removed post translationally in vivo, the purified hemoglobin has a normal N-terminal valine. For this molecule, P₅₀=23 to 25 and N=2.5 when measured at 37°C. Thus, in the above instances, the replacement of an N-terminal valine with an N-terminal methionine increased the P₅₀ value. Under physiological conditions, it is expected that the genetically fused Presbyterian hemoglobin produced in E. coli will deliver 20-30% more oxygen than the similar hemoglobin, with its altered N-terminus, produced in yeast.

High Affinity Mutants

The term "high affinity hemoglobin-like protein" refers to a hemoglobin-like protein having a P_{50} which is at least 10% less than the P_{50} of cell free hemoglobin A_0 under the same conditions.

High affinity mutant hemoglobin may have utility in certain situations. For example, perfluorocarbon-based blood substitute preparations are under clinical study for enhancement of radiation therapy and certain chemotherapy treatments of solid tumors (Dowling, S., Fischer, J.J., and Rockwell, S. (1991) Biomat. Art. Cells Immobil. Biotech, 19, 277; Herman, T.S. and Teicher, B.A. (1991) Biomat. Art. Cells and Immobil. Biotech, 19, 395; Holden, S.A., Teicher, B.A. and Herman, T.S. (1991) Biomat. Art. Cells and Immobil. Biotech, 19, 399.) The basis of these investigations is the fact that oxygen is a required component of the cell toxicity action of radiation and certain chemotherapy reagents. Solid tumors frequently exhibit extremely low partial oxygen pressure in the interior of the tumor, rendering therapy inefficient. Perfluorocarbon-based oxygen-carrying solutions appear to dramatically enhance certain tumor therapies, and hemoglobin-based blood substitutes are expected to have a similar utility. It is likely that cell-free hemoglobin unlike whole red blood cells, will be able to penetrate the interior region of tumors for delivery of oxygen. Actual percent of oxygen released by a cell-free hemoglobin preparation is not a direct function of P_{50} but rather depends on the shape of the oxygen equilibrium binding curve between the two pressures representing the partial oxygen pressure of the lungs (where oxygen is loaded onto hemoglobin) and the partial pressure of the tissue where oxygen is unloaded. Therefore, it is possible that a high

affinity mutant hemoglobin would be preferred as a tumor therapy adjuvant. A high affinity hemoglobin would retain its bound oxygen throughout the normal circulatory system, where partial oxygen pressure remains relatively high, but release its oxygen in the extremely oxygen-depleted tumor interior. Normal or low affinity hemoglobin might have less hemoglobin available for release by the time it reaches the interior of the tumor.

Naturally occurring high affinity hemoglobin mutants are also known, see Bunn and Forget, Table 14-1, and candidate non-naturally occurring high affinity hemoglobin mutants may be proposed in view of the known mutants and hemoglobin structure. Particularly preferred high affinity mutants are set forth in Table 400.

It should be noted that genetic fusion and crosslinking can affect oxygen binding affinity.

Cysteine Mutations and Disulfide Bridge Formation

Cysteine mutations are of value for increasing the stability of the tetramer (See USP 5,028,588 and Ser. No. 07/443,950. They also facilitate constructing poly(tetrameric) ($n \geq 2$) hemoglobins. This is because the cysteines on adjacent tetramers (including pseudotetramers) can be oxidized to form a disulfide bridge, covalently coupling the tetramers. In addition, the thiol groups of cysteines may be reacted with a variety of crosslinking agents.

A variety of sites are available for introduction of cysteines into a hemoglobin-like protein.

The criteria governing site selection are: (1) the mutation does not affect functionality; (2) the side chain is accessible to water in oxy or deoxy structure; (3) the site should lie on the surface of the folded protein;

(4) the sulfhydryl of the side chain should extend away from the surface rather than toward the interior of the molecule; (5) the site should be in a portion of the molecule that is not directly involved in the R->T transition; (6) the change should be in a portion of the molecule that does not have a tightly fixed position (such regions generally give indistinct X-ray diffraction patterns); (7) the mutations will not destroy the local secondary structure, i.e., avoid pro->cys mutations, which might result in a refolding problem; and (8) if possible, a conservative change should be made such as ser->cys or ala->cys. A mutation does not necessarily have to meet all of the above requirements to be useful. For example, one might envision a site that is involved in the R->T transition (cf. 5 above) but confers a beneficial change in P₅₀ (cf. 1 above) because of that involvement. The most important considerations are that the mutation does not abolish O₂ binding, before or after crosslink formations, and that the cysteine is accessible for participation in the desired crosslinking reaction.

Candidate sites on the alpha surface include:

his72, asn 78, asn68, ala71, thr67, lys7, lys11, thr8, ala12, thr118, lys16, ala45, glu116, gly15, his112, thr24, glu23, lys60, lys56, his50, gly51, glu53, ser49, asp47, gln54, his45, lys90, ala82, lys61, ala19, his20, asp85, ser81, asp75, asp74, lys139, asp64, and gly18 (total 40 amino acids).

Candidate sites on the beta surfaces includes:

asp79, his2, leu3, thr4, glu6, ser9, thr12, ala13, gly16, lys17, val18, asn19, val20, asp21, glu22, lys65, ser72, ala76, his77, asp79, asn80, gly83, ala86, thr87, glu90, lys95, lys59, glu43, ser44, asp47, ser49, thr50, ala53, asp52, lys61, glu121, lys120, thr123, lys66, asp73, ala62, his116, his117 (total 45 amino acids).

There are a number of naturally occurring mutants which already show mutations at these sites. These are listed below:

	Residues	Region	Mutation
5	19	AB1	ALA->GLU ALA->ASP
	54	E3	GLN->ARG GLN->GLU
	71	E20	ALA->GLU
10	75	EF4	ASP->GLY ASP->HIS ASP->TYR ASP->ASN
	81	F2	SER->CYS
15	47	CE5	ASP->GLY ASP->HIS ASP->ASN

If the pseudo-octamer (n=2) is formed by directly linking two pseudo-tetramers via a disulfide bond, the halflife in serum may be influenced by the rate at which endogenous serum small molecule thiols (such as glutathione) reduce the disulfide bond. The mechanism of these reactions involves the thiolate anion as the actual reducing species (Creighton, T.E. (1978) Prog. Biophys. Molec. Biol., 33:259-260; Creighton, T.E. (1975) J. Mol. Biol., 96:767; Creighton, T.E. (1977) J. Mol. Biol., 113:313). Thus the rate of reduction will be a function of

the molecular electrostatic environment in the vicinity of the disulfide bond. A slower rate of reduction would be predicted if the disulfide was located in an electrostatically negative environment,, due to the repulsion of the thiolate anion. In the case of glutathione, even the unreactive transient protonated species has a net negative charge and would be repulsed, thus further reducing the rate of disulfide reduction.

A surface or near-surface amino acid residue of di- alpha or di-beta hemoglobin that is located in close proximity to a negatively charged surface residue might therefore be a good choice for location of a single cysteine mutation in the di-alpha or di-beta polypeptide. Although formation of the initial disulfide bond between two such cysteines might also be slower because of repulsion between the negative charges on the two hemoglobin molecules in the vicinity of the cysteines, the reaction could be facilitated by use of high salt or high pH during the in vitro bond formation reaction. If carried out under deoxy conditions in a redox buffer, the reaction might also be facilitated by temperature elevation.

Preferred sites for cys mutations proximal to negative charged residues

alpha ser49	near asp47; naturally occurring ser49 to arg has normal O ₂ affinity
alpha his20	near glu23; naturally occurring his20 to tyr, gln, arg have no known undesirable properties
alpha lys16	near glu16; naturally occurring lys to glu has normal O ₂ affinity
alpha his50	near glu30; naturally occurring his50 to asp has no known undesirable properties
beta thr50	near asp52; naturally occurring thr50 to lys has no known undesirable properties
beta lys65	near asp21
beta asn19	near asp21

Surface or near-surface cysteine mutations in general are not expected to have major effects on the functionality of the hemoglobin pseudotetramer. Cysteine mutations would not be expected to significantly destabilize alpha helices, and surface residues are not directly involved in the oxygen binding properties of hemoglobin. Most surface residues undergo considerable motion and are not tightly constrained. It should also be noted that because of protein breathing motions, the cysteine side chain would not necessarily have to point directly into solution to be accessible for disulfide bond formation.

In addition to the use in construction of a pseudo-octamer, there may be additional uses of surface cysteine mutations. These include: (1) construction of multimeric hemoglobins ($n > 2$) by use of synthetic sulfhydryl reactive peptides with more than two reactive sites; (2) surface cysteine residues could be used to attach chelates that bind radioisotopes for imaging; and (3) surface cysteines could be used to attach bio-active peptides or other therapeutic agents to increase their circulating half-life, or target their delivery. If the attachment of the drug were via a disulfide, the rate of release of the peptide from its carrier could be controlled by neighboring residues. For uses (2) and (3), restriction to one cysteine per di-alpha or di-beta is unnecessary.

It may be desirable to eliminate the cysteine at beta 93 of normal human hemoglobin so that it cannot participate in polymerization reactions. This cysteine may be replaced by serine, alanine or threonine, for example. Other wild-type cysteines may also be replaced, if desired, but it is unlikely that they participate in crosslinking reactions after the tetramer is formed.

Mutations to Reduce Haptoglobin Binding

It is presently believed that haptoglobin binding plays a role in the catabolism of hemoglobin. If so, intravascular retention of hemoglobin might be enhanced by mutations which inhibit haptoglobin binding. Oxyhemoglobin dissociates into alpha-beta dimers, which are then bound by haptoglobin. While much of the binding energy is associated with binding to residues which are buried in the tetramer but exposed in the dimer, it appears that there are also secondary binding sites on the surface of the tetramer. Though the mechanism is not clear, the haptoglobin-bound dimers are transported to Kupffer cells in the liver, where they are catabolized.

It would be most desirable to mutate sites which both are involved in haptoglobin binding and which are suitable for attachment of another tetramer. Candidate mutation sites are in the alpha chain of normal human alpha globin, residues 1, 6, 74, 82, 85, 89, 90, 93, 118, 120-127 and 139-141, and in the beta chain, residues 2, 11-40 and 131-146. It is unlikely that haptoglobin binding can be blocked merely by single substitution mutation of one genetically encoded amino acid to another. However, if the above residues are replaced by a cysteine, and the cysteine is crosslinked to another molecule which is significantly larger than the usual amino acid side chain, the steric effect is magnified considerably and haptoglobin binding may be inhibited. Of course, to retain polymerization control, these mutations should be made asymmetrically in a pseudooligomeric polypeptide so that there is only one crosslink per tetramer.

It is known that even covalently crosslinked hemoglobins can be processed by haptoglobin; this is thought to be the result of the "breathing" of the tetramer

in its oxy form sufficiently to allow the haptoglobin access to the normally buried residues of the subunit interfaces in question. This may be prevented by tightly crosslinking the globin subunits so dissociation will not occur within the time span of interest. Unlike the mutations discussed above, these mutations should be made in all of the indicated subunits for maximum efficiency.

beta37->Cys and alpha92->Cys
beta40->Cys and alpha92->Cys
beta97->Cys and alpha41->Cys

The above mutations all lie at the $\alpha_1\beta_2$ and $\beta_1\alpha_2$ interfaces and lock these interfaces shut so that "breathing" does not allow haptoglobin access.

"Breathing" may also be inhibited by low oxygen affinity mutations; the tetramer then spends more time in the deoxy state, which is not susceptible to haptoglobin attack.

Pseudomeric Globin-Like Polypeptides and Pseudotetrameric Hemoglobin-Like Proteins Useful as Intermediates in Preparation of Multimeric Hemoglobin-Like Proteins

In the liganded form, haemoglobin readily dissociates into $\alpha\beta$ dimers which are small enough to pass through the renal glomeruli, and Hb is thereby rapidly removed from the circulatory system. Intravenous administration of haemoglobin in amounts far less than that needed to support oxygen transport can result in long term kidney damage or failure. Ackers, G.K. and Halvorson, H.R., Proc. Nat. Acad. Sci. (USA) 71, 4312-16 (1974); Bunn, H.F., Jandl, J., J. Exp. Med. 129, 925-34 (1969). If

dissociation into dimers is prevented, there is an increase in intravascular half life and a substantial reduction off renal toxicity. Lee, R., Atsumi, N., Jackbs, E., Austen, W., Vlahakes, G., J. Surg. Res. 47, 407-11 (1989). The hemoglobin-like proteins of the present invention cannot dissociate into $\alpha\beta$ -dimers without the breakage of a peptide bond and should have the advantages of a longer intravascular half life and reduced renal toxicity.

In the crystal structures of both deoxyhaemoglobin and oxyhaemoglobin the N-terminal Val residue for one α subunit and the C-terminal Arg residue of the other α subunit are only between 2 and 6 Å apart, and are bound to one another through a salt bridge in deoxyhaemoglobin. Fermi, G., Perutz, M., Shaanan, B., Fourme, R., J. Mol. Biol., 175, 159-74 (1984); Shaanan, B., J. Mol. Biol. 171, 31-59 (1983). This distance could be spanned by one or two amino acids. One extra amino acid can be added to the C-terminal Arg residue of the α subunits by trypsin catalyzed reverse hydrolysis without significantly altering the oxygen binding properties. Nagai, K., Enoki, Y., Tomita, S. and Teshima, T., J. Biol. Chem., 257, 1622-25 (1982) Preferably the di-alpha linker (if one is used) consists of 1 to 3 amino acids which may be the same or different. A Mono-Gly linker is especially preferred. In designing such a linker, it is important to recognize that it is desirable to use one or more amino acids that will flexibly connect the two subunits, transforming them into domains of a single di-alpha globin polypeptide.

The preparation of "di-beta" mutants is also contemplated. The distance between the N-terminus of one beta subunit and the C-terminus of the other is 18.4 Å in the deoxy configuration and 5.2 Å in the oxy form. Preferably, the di- beta linker consists of 2 to 9, amino

acids which may be the same or different. Glycine amino acids are particularly preferred.

5 The length of the $(\text{-gly-})_n$ genetically fused link between the N-terminus of one beta chain (at β_1 , 1 Val) and the C terminus of the second beta chain (β_2 , 146 His) in di- beta hemoglobin may range between 1 and approximately 9 glycines. In the oxy and deoxy crystal structures of human hemoglobin A_0 , the distance between these termini is 5.22 Å and 17.93 Å respectively (from the N-terminal nitrogen to the C terminal carbon of the carboxylate). A single glycine linker, which is a little less than 4 Å in length, may come close to linking the two termini in the oxy structure, however, it is expected that this linker will fall ~ 14Å short in the deoxy structure. 10
15 Significantly more perturbation of the deoxy structure vs the oxy structure might be anticipated with this linker. Some alterations in the oxygen binding properties may be caused by deletion of the positive and negative charges at the two termini and their inclusion in the amide bond. In addition, the linker molecule itself may destabilize the oxy structure less than the deoxy structure, and thus lead to a relative increase in oxygen affinity. Likewise, two glycines inserted as linkers may also differentially stabilize the oxy structure and hence relatively increase the oxygen affinity by the same mechanism described above. 20
25

30 When the number of linking glycines is increased to 5, the linker should just span the cleft between the beta chain termini in the deoxy structure, and, moreover, insert added steric bulk between the termini in the oxy structure, thus leading to a relative stabilization of deoxy (or destabilization of oxy) and perhaps resulting in a concomitant decrease in oxygen affinity. Due to the large space between the beta termini in the deoxy (but not the oxy structure), addition of glycine linkers in the

range of 6-9 may further stabilize the oxy structure and, in the same manner, further decrease oxygen affinity.

5 A third form of globin pseudodimer is one comprising both alpha and beta globin domains. A possible route to fusing alpha1 to beta2 and so stabilizing hemoglobin against $\alpha_1\beta_1/\alpha_2\beta_2$ dimer formation, is to fuse the alpha1 C-terminal residue to the N-terminal residue of beta2 C helix, creating a new C-terminus at the end of the beta2 B helix. The original beta N terminus, Val1, would be fused to the original beta subunit C-terminal residue, His146, by means of an intervening new section of protein, thus creating a continuous polypeptide chain comprising the alpha and beta subunits of different dimers. This chain may be described as follows: $\alpha(1-14)-\text{Gly}_3-\beta(35-146)-\text{Gly}_{1-3}-\text{Ala}_{13}-\text{Gly}_{1-3}-\beta(1-34)$; See Figure 6.

10 Inspection of the structure of human deoxyhemoglobin using a molecular graphics computer indicates the following relevant distances. The distance between the Alpha1 Arg141 carboxyl carbon and Beta2 Tyr35 N atoms is approximately 8.6 Angstroms. A fully extended linear triglycine peptide measured approximately 10.1 Angstroms from the N to C terminal residues. This suggests that three glycine residues could be employed to span the distance between the Arg141 and Tyr35 residues with a minimum of unfavorable steric interactions and maximum conformational freedom. The distance requirements could be different in oxyhemoglobin, and if so, the sequence of the fusion peptide could be altered to best accommodate the requirements of both structures.

25 In human deoxyhemoglobin, the distance between the Beta2 His146 carboxyl carbon and the Beta2 Val1 nitrogen atoms is approximately 25 Angstroms. A right handed 3.6 Alpha helix constructed from a linear sequence of 13 Alanine residues was found to measure 22 Angstroms

from N to C terminus. With the addition of one to three glycine residues at each end of this helix (to give $\text{Gly}_n(\text{Ala})_{13}\text{Gly}_n$ where $n=1$ to 3), it could span the required distance and have sufficient conformational flexibility to avoid serious tertiary packing conflicts. Additionally, the amino acid sequence of the helix could be altered to introduce favorable hydrogen bonds and salt bridges between the new helix and the Beta2 helix against which it would pack in the folded protein. Such interactions could aid stabilization of the engineered protein.

Glycine is the preferred amino acid in the linkers, since it is known to be quite flexible, Cantor and Schimmel, Biophysical Chemistry, part 1, pp. 266-9 (1980), and also allows chains into which it is incorporated to assume a more compact structure. However, the residues comprising the linker are not limited to glycines; other residues may be included instead of or in addition to glycine, such as alanine, serine, or threonine. Since these amino acids have a more restricted conformational space in a protein, they will likely result in more rigid linking chains, and hence have a more pronounced effect on the relative stabilization/destabilization of the oxy/deoxy structures.

It should be understood that the minimum and maximum number of amino acids in the linker is a function of the distance to be spanned in both the oxy or deoxy forms, the amino acids chosen, and the propensity of the particular amino acid sequence to form a secondary structure. While a random coil is usually preferred, it is not required, and a linker with a large number of amino acids in a secondary structure may have the same span as a random coil linker with fewer amino acids. A linker may comprise, e.g., 1-3 glycines, followed by a sequence having a secondary structure, followed by 1-3 more glycines. The

translation per residue, in angstroms is 1.9 for polyproline I, 3.12 for polyproline II, 3.1 for polyglycine II, 3.4 for an antiparallel β sheet, 3.2 for a parallel β -sheet, 1.5 for a right handed α -helix, 2.0 for a 3_{10} helix, and 1.15 for a π helix. In a fully extended chain, the maximum translation per residue is 3.63 Å if the repeating units are staggered and 3.8Å if the peptide bond is trans.

The number of amino acids in the linker may be such that a formation of a secondary structure, such as an alpha helix or a beta-sheet, is undesirable, as the span is reduced. Certain amino acids have a greater tendency to participate in such structures. See Chou and Fasman, Biochemistry, 13:222-245 (1974), incorporated by reference. The amino acids are ranked in order of decreasing participation below. The preferred linker amino acids are boldfaced. Glycine is the most suitable amino acid for this purpose. The most preferred di-alpha linkers are Gly or Gly-Gly.

	<u>Alpha Helix</u> <u>Formers</u>	<u>Beta Sheet</u> <u>Formers</u>
	Glu (1.53)	Met (1.67)
	Ala (1.45)	Val (1.65)
5	<u>Leu (1.34) Hα</u>	<u>Ile (1.60) Hβ</u>
	His (1.24)	Cys (1.30)
	Met (1.20)	Tyr (1.29)
	Gln (1.17)	Phe (1.28)
	Val (1.14)	Gln (1.23)
10	Trp (1.14)	Leu (1.22)
	<u>Phe (1.12) hα</u>	Thr (1.20)
	<u>Lys (1.07)</u>	<u>Trp (1.19) hβ</u>
	Ile (1.00)	<u>Ala (0.97) Iβ</u>
	<u>Asp (0.98)</u>	<u>Arg (0.90)</u>
15	Thr (0.82)	<u>Gly (0.81)</u>
	<u>Arg (0.79)</u>	<u>Asp (0.80) iβ</u>
	<u>Ser (0.79)</u>	Lys (0.74)
	<u>Cys (0.77) iα</u>	<u>Ser (0.72)</u>
	<u>Asn (0.73)</u>	His (0.71)
20	<u>Tyr (0.61) bα</u>	<u>Asn (0.65)</u>
	<u>Pro (0.59)</u>	<u>Pro (0.62) bβ</u>
	<u>Gly (0.53) Bα</u>	Glu (0.26) B β

(The letter symbols are H α , strong α former; h α , α former; I α ; weak α former; i α , α indifferent; b α , α breaker; and B α strong α breaker. The β symbols are analogous. Trp is b β if near the C-terminal of a β -sheet region.)

The alpha helix of a polypeptide chain comprises an average of 3.6 residues per turn. In globular proteins, the average length is about 17Å, corresponding to 11 residues or 3 helix turns. In alpha and beta globin, the helices range in length from 7 to 21 amino acids (A.A.). The beta pleated sheet comprises 2.3 residues per turn; the average length is about 20Å or 6 residues.

Chou and Fasman define an alpha helix nucleus as a hexapeptide containing four helix forming residues and not more than one helix breaker, and a beta sheet nucleus as a pentapeptide containing three beta sheet forming residues and not more than one sheet breaker.

The amino acid sequence in the vicinity of the di-alpha linker is as follows:

residue #	138	139	140	141		1	2	3	4
AA	Ser	Lys	Tyr	Arg	-(XXX) _n -	Val	Leu	Ser	Pro
5 (SEQ ID NO:4)									(SEQ ID NO:5)
Helix Not	H21	HC1	HC2	HC3		NA1	NA2	A1	A2
Helix Pot	079	107	061	079		114	134	079	059
Sheet Pot	072	074	129	090		165	122	072	062

10 (Note: Helix- and sheet forming potentials have been multiplied by 100 for typographical reasons.)

The di-alpha linker is preferably only 1-3 amino acids. Thus, it can form an alpha helix only in conjunction with the linker "termini". A one or two residue linker, even if composed
15 of amino acids with strong secondary structure propensities, would be unlikely to assume an alpha helix or beta sheet configuration in view of the disruptive effect of, e.g., Arg 141 or Ser 3. If the linker is 3 residues long, it would be preferable that no more than one residue be a strong alpha helix
20 former, unless the linker also included a strong alpha helix breaker.

The amino acid sequence in the vicinity of the di- beta linker may impose more stringent constraints.

143	144	145	146		1	2	3	4
25 His	Lys	Tyr	His	-(XXX) _n -	Val	His	Leu	Thr
(SEQ ID NO:6)					(SEQ ID NO:7)			
H21	HC1	HC2	HC3		NA1	NA2	NA3	A1
124	107	061	124		114	124	134	082
071	074	129	071		165	071	122	120

30 The di-beta linker is likely to be longer (preferably 1-9 A.A.) and therefore more susceptible to secondary structure formation. If secondary structure formation is not desired, it is desirable that the amino acid adjacent

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to Val-1 be an alpha helix breaker (e.g., Glycine) in view of alpha-helix propensities of Val-His-Leu. More generally, it is desirable that the linker not contain (or cooperate with the proximately linked amino acids to form) an alpha helix nucleus or beta sheet nucleus.

When secondary structure is not desired, amino acids with a high propensity toward alpha helix formation may be used in the linker if accompanied by "helix breaking" amino acids. Similarly, Beta sheet formation may be prevented by "sheet disrupting" amino acids.

Of course, prediction of secondary structure using Chou and Fasman's approach has its limitations and the ultimate test of the acceptability of a linker is whether or not the di-alpha or di-beta hemoglobin has the desired affinity for oxygen. In particular, a poly-alanine linker, despite its supposed propensity to alpha-helix formation, may well be of value since the alanine group is compact and therefore the linker should be quite flexible if secondary structure does not form.

In an especially preferred embodiment, di-alpha and beta globin genes are combined into a single polycistronic operon. The use of a polycistronic operon is not, however, necessary to practice the present invention, and the alpha (or di-alpha) and beta (or di-beta) globin genes may be expressed from separate promoters which may be the same or different.

While the preferred "genetically fused hemoglobin" of the present invention is one comprising a di-alpha and/or di-beta globin, other globin chains may be genetically fused and used in the production of multimers of hemoglobins of species other than Hgb A1 ($\alpha_2\beta_2$).

Pseudo-Octameric (Ditetrameric) Hemoglobin-like Proteins With Disulfide Bridges

The ability to produce pseudotetrameric recombinant hemoglobins consisting of a single dialpha polypeptide and two beta chains (or a dibeta polypeptide and two alpha chains) provides a unique opportunity to create an asymmetric pseudotetramer from the normally symmetric pseudotetramer. Because the two alpha globin domains are expressed as a single polypeptide, it is possible to alter one of the alpha globin domains without altering the other. The result is a protein that, in its final folded state, contains two different alpha globin domains in a strict 1:1 ratio. This type of asymmetric hemoglobin molecule, with its unique chemical properties, cannot be easily constructed by any other method. A preferred embodiment of this invention would involve use of site-directed mutagenesis to substitute a cysteine residue in one of the two alpha globin domains of a di-alpha hemoglobin such as SGE1.1 (a di-alpha hemoglobin with a beta chain Presbyterian mutation) such that the cysteine would be on the surface of the folded recombinant hemoglobin molecule. A homogeneous preparation of pseudo-octameric hemoglobin could then be formed through interhemoglobin linkage of two pseudotetramers either directly by simple oxidation of purified pseudotetramers or by reaction with a bridging molecule (Figure 3).

Although direct formation of a disulfide bond between two "mono cys" tetramers is desirable in order to avoid the need for chemical crosslinking, naturally occurring reducing agents may reduce the disulfide bond in vivo at a significant rate. Preliminary experiments suggest that the rate of reduction of the bond may be influenced by the location of the cysteine mutation on the surface of the hemoglobin.

The surface cysteine mutants (MW = 64 kDa) can be oxidized to the disulfide-linked dimer under oxidative

conditions. This can be accomplished by stirring a concentrated solution of the expressed protein at pH 8 under pure oxygen at 4°C or room temperature in the dark. Trace levels of transition metal ions such as Cu^{+2} may be added to level below 1 uM to catalyze the oxidation (1). Formation of the 128 kDa octamer can be monitored by gel filtration. Saturation of the solution with oxygen at elevated pH should minimize autooxidation of recombinant hemoglobin.

An alternative procedure, which may be the preferred method of catalyzing this reaction, involves the use of redox buffers such as reduced and oxidized glutathione, or reduced and oxidized dithiothreitol (2). This catalysis of the reaction through disulfide interchange may be necessary to control trace transition metal catalysis (3). An second, similar approach involves conversion of the surface cysteines in the 65 kDa species to sulfonates before purification (to avoid 128 kDa species formation during purification), followed by conversion to the disulfide-linked 128kDa species with reduced glutathione (2).

(1) Freedman, R.B. and Hillson, D.A. (1980) "Formation of Disulfide Bonds" IN: The Enzymology of Post Translational Modification of Proteins, Vol. 1, p. 157 ff. (Academic Press).

(2) DiMarchi, R., et al. (1988) Chemical synthesis of human epidermal growth factor (EGF) and human type a transforming growth factor (TGFA) IN: Peptides: Chemistry and Biology (G.R. Marshall, ed.) pp. 202-203 (Leiden:ESCOM).

(3) Creighton, TE (1978) Experimental studies of protein folding and unfolding. Prog. Biophys. Molec. Biol. 33:231-297

Multimeric Hemoglobin-Like Proteins With Other Intercysteine Linkages

5 It is also possible, of course, to couple two mono cys molecules with a homobifunctional crosslinking reagent resulting in linkage via nonreduceable bonds. The degree of polymerization is still controlled by the use of the
10 mono cys di-alpha or di-beta Hgb starting material.

By using bi-, tri-, tetra-, hexa-, or octa-functional crosslinkers several properties of multimeric hemoglobin which may contribute to longer serum half life can be controlled. The crosslinkers can be designed to give a
15 nonreducible bond between two tetramers, to yield high molecular weight multimers of $n > 2$ psuedo-tetramers (e.g. dodecamers, etc.) and/or to drop the overall isoelectric point of a hemoglobin octamer to further increase its half life.

20 Correlations of molecular weight with serum half life for proteins such as IL-2, demonstrate that a significantly longer half life may be expected as the molecular weight of a protein increases, particularly above the renal filtration limit of 50-70 kDa. However,
25 a factor potentially limiting the half life of multimeric hemoglobin formed by a disulfide link between tetramers is reduction of the cys-cys disulfide bond by endogenous thiol-reducing agents found in the serum. Estimates of small molecule thiol levels in plasma vary from 17 μ M to
30 5 μ M. The major species is reduced glutathione. Other thiol compounds in plasma include cysteine, homocysteine, and gamma-glutamyl cysteine. Thus, small molecule plasma

thiols are available for reduction of disulfide bonds. This may be reflected in the diminished half life seen with antibody-ricin A chains conjugates linked by regular disulfides (6.7 hrs) relative to conjugates linked with sterically hindered, and thus less reducible, alpha-methyl disulfides (42.5 hours).

Thus, in one embodiment, the octameric hemoglobin features a nonreducible sulfur-containing crosslink such as a thioether bond or thiol-maleiimide adduct. These may substantially extend the multimer half life. Simple homobifunctional crosslinkers or polyethylene glycol (peg) derivatives would likely be useful for this purpose (see below). The reaction of a bifunctional cysteine-specific crosslinker with a mono-cys di-alpha or di-beta Hgb should limit the products of the reaction to a dumbbell-like octameric hemoglobin and unreacted hemoglobin. The reaction should be stoichiometric when the Hgb and crosslinker are present at high concentrations and the Hgb is present in a slight excess over the crosslinker maleiimides at pH 6.5-7.0. Further, there should not be substantial interference by reaction with globin lysines. The preferential reactivity of the thiols to lysines can be roughly calculated as the product of their molar ratios and the ratio of the intrinsic reactivity of a maleiimide to thiols versus amines. This product is ca. $[1 \text{ cys}/40 \text{ lys}] \times [1000] = 25$ at pH 7. The side products would still be octamers, with one attachment site being a secondary amine and thus might well be functionally equivalent to the S-crosslinked octamers. Hydrolysis of the maleiimide adduct at pH 7 would be slow, and the ring opening would leave the crosslink intact.

The reaction of the thioether $\text{RC}(=\text{O})\text{CH}_2\text{I}$ with the sulfhydryl-bearing protein ($\text{R}'\text{SH}$) results in the crosslink $\text{RC}(=\text{O})\text{CH}_2\text{-S-R}'$. The reaction of the maleiimide with the

protein results in the addition of the R'SH across the double bond of the five-membered maleiimide ring, yielding a thiomaleiimide adduct.

The following are examples of homobifunctional crosslinkers that may form metabolically stable crosslinks between monocysteine pseudo tetramers:

- 1) 1,2-bis-(2-iodoethoxy)ethane
- 2) 4,4'-dimaleiimidylbenzene or N,N'-p-phenylenedimaleiimide
- 3) N,N'-bis-(3-maleiimido-propionyl)-2-hydroxy-1,3-propane diamine.

Longer half lives may also be obtained by increasing the apparent solution molecular weight by simply lengthening the distance between the two linked tetramers using a long crosslinking agent. The use of some potentially novel polyethylene glycol derivatives as homobifunctional crosslinkers, reacting with SGE1.1 monocys, may provide one mechanism for significantly increasing the molecular weight of octameric hemoglobin by virtue of the length of the crosslinker alone.

A suitable crosslinker for this purpose is



The length may be adjusted by variation of n . A few examples are given below.

<u>Structure</u>	<u>Max Length</u>	<u>Source</u>
$n = 22$	$\sim 49\text{\AA}$	peg -1000
$n = 76$	$\sim 166\text{\AA}$	peg -3350
$n = 227$	$\sim 499\text{\AA}$	peg -10000

Homobifunctional N-hydroxysuccinimide-activated peg has been used previously to derivatize hemoglobin. Yabuki, et al., Transfusion, 30:516 (1990). This reaction resulted in a polydisperse mixture of monomeric, dimeric, and trimeric species with an average stiochiometry of peg/hemoglobin of 6.2. However, 83% of the hemoglobin derivatized by peg was not crosslinked to another hemoglobin molecule. Control of the peg-derivitization of wild-type hemoglobin was not possible because there is no site-directed labeling of the hemoglobin starting material.

In contrast, the combination of SGE 1.1 mono-cys starting material and a peg crosslinker should yield a substantially monodisperse dumbbell (pseudo-octameric) product. The site-direction of the crosslinker attachment site should result in precise control of the apparent molecular weight, which will depend on the size of the crosslinker. Moreover, careful control of the site of the cys mutation on the surface of the recombinant hemoglobin should ensure that the functionality of the derivatized hemoglobin is maintained.

Higher Multimeric Hemoglobins

The above crosslinkers all involve the attachment of one tetramer at each end of a crosslinker. It may be advantageous to attach more than two tetramers to a single crosslinker to yield more oxygen-carrying capacity and to further increase the molecular weight.

A multimeric hemoglobin may be assembled with the aid of one or more linker peptides, each having a controlled number of reactive sites to which a cysteine residue of a hemoglobin tetramer or pseudotetramer may be attached, directly or indirectly.

With a peptide linker of considerable length, there is the concern that it will be degraded by serum proteases, thus degrading the multimeric hemoglobin into its component tetramers. This problem, if significant, may be remedied by use of a peptide linker which is less susceptible to proteolysis. A non-exhaustive list of such linkers would include peptides composed of D-amino acids, peptides with stable, extended, secondary structures, and branched peptides.

In the case of peptides composed of D-amino acids, use of D-Glu or D-Asp is particularly preferred.

A number of stable, extended, secondary structures are known. The simplest is possibly polyproline. Another example is the 2-stranded coiled coil, in which two peptide chains intertwine. A 4-helical or a 4-stranded coiled coil are also possibilities.

Branched structures, such as those obtained by derivation of the secondary amino group of lysine, are typically resistant to protease.

If desired, several of these approaches may be combined. For example, several coiled coils may lead off a branched structure, or D-amino acids may be incorporated into a coiled coil.

A hypothetical 4- tetramer coiled-coil linker complex is shown in Figure 4(a). Design and synthesis of these coiled coil peptides has already been explored (for an example see Cohen and Perry, Proteins, 7:1-15 (1990)). The rationale for a coiled coil is that two intertwined alpha helices will be less sensitive to proteolytic cleavage than a single naked secondary structure like an extended peptide (rapidly cleaved by proteases), an alpha helix or a beta sheet.

Using molecular modeling, an internal disulfide may be designed in the center of a bi-functional coiled coil

linker such that the strands are covalently attached. This should stabilize formation of the correct coiled coil crosslinker before mono-cys di-alpha or di-beta Hgb (e.g., sge1.1 cys) is attached. Additionally, a tri-functional crosslinker can be stabilized by use of a orthogonally-protected lysine (lys-FMOC) rather than a disulfide in the center of a proteolytically inert secondary structure. A polyproline helix can be used as the linker, and can be stabilized by branching the synthesis at the lys-FMOC after removal of the side chain. The three remaining lysines in the branched peptide would then be iodoacetylated to site-specifically attach a thiol-reactive group using either iodoacetic anhydride or N-succinimidyl-iodoacetate and subsequently reacted with sge1.1-cys. An analogous tetra-functional crosslinker could be synthesized by inserting 1-2 prolines between two internal branching lysines to rotate them such that the two internal branching chains growing off the orthogonally protected lysines head in (nearly) opposite directions. Analogous structures could be made using D-glutamate(E) or D-aspartate(D) to provide protease resistance, and these would form an extended polyanionic chain at pH 7.

The sequence of a hypothetical alpha-helical coiled coil is modified from that given in Semchuck, et al., in Peptides: Chemistry, Structure and Biology; 566 (Rivier and Marshall, eds:1990), to leave only two lysines (K) at each end:

ACKCAELEGRLEALEGRLEALEGRLEALEGRLEALEGKL-amide (SEQ ID NO:8)

This coiled coil should have about 10 turns of a helix and thus will be ca. 54 Å long, allowing two tetramers to attach on each side without steric interference. The exact sequence and length to allow appropriate placement

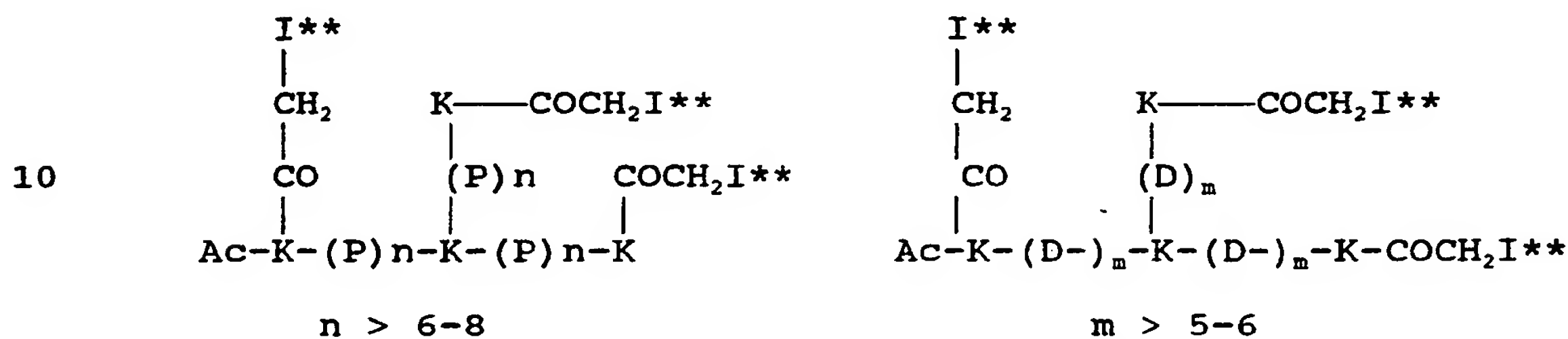
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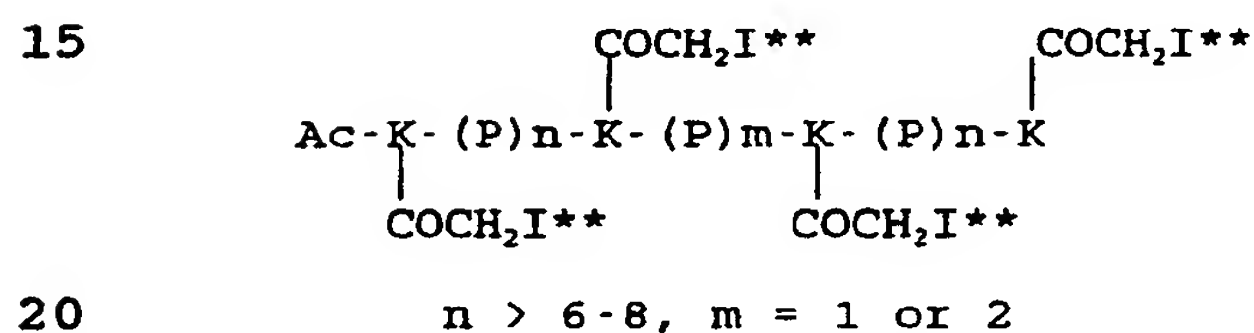
of 4 tetramers would depend on the results of molecular modeling.

Suggested trifunctional and tetrafunctional crosslinkers are diagrammed below.

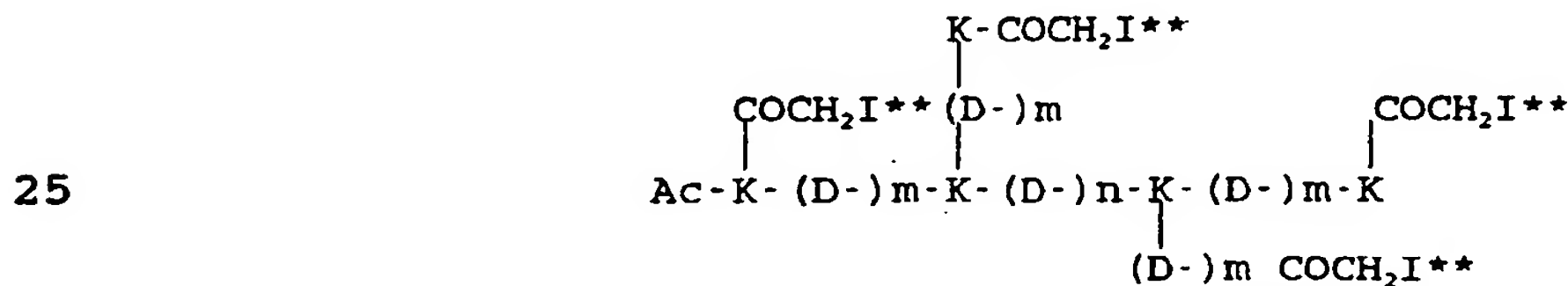
5

TRIFUNCTIONAL

15

TETRAFUNCTIONAL

20



25

$m > 5-6, n = 1 \text{ or } 2$

30

(D could be a D-amino acid for greater protease resistance)
 (** indicates a reactive site; K is a lysine; P is Proline, Ac is acetyl; I is iodine; D is D- or L-aspartate; K's are on opposite

58.

faces so the pseudotetramers attach on opposite faces of the coiled coil.)

See also Figure 4(a).

Another possibility is an 8-hemoglobin complex (Figure 4(b)). The rationale for considering this sort of complex is that it may be the way to obtain a very long half-life, due to the extreme stability of the "crosslinker" and the substantially higher molecular weight of the complex. The crosslinker might take the form of a doubly branched coiled coil, with a Lys(FMOC) replacing an Arg in the middle of the chain to allow the branching, and with a polyproline helix or other protease resistant secondary structure comprising the branching moiety. This structure could allow attachment of 6 SGE1.1's per crosslinker. Alternatively, a 4-helical bundle protein (See Figure 4(c)) or 4-stranded coiled-coil such as those synthesized by DeGrado, Science, 243:622 (1989), with each helix in the 4-helical bundle containing the consensus sequence GELEELLKKLKELLKG, (SEQ ID NO:9) the helices being linked by three PRR or RPR loops, could be utilized as a suitable core for the linker. This is one of the most stable proteins known, with a $G = -22$ kcal/mole separating the folded from the unfolded state. Each helix would be 4+ turns or ca. 22 Å long. Since this may not be enough room to fit two hemoglobins with one anchored at each end of the helix, they might have to be attached to different faces of the same helix, to lysines placed at each end of the polar face of each helix. Each helix is amphipathic; this should allow relative freedom to have a total of 8 lysines (and no more) and to change the remaining lysines to arginines. At least two of the $i, i+4$ salt bridges per

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helix would be retained for stability of the protein. Attachment of an externally crosslinkable cysteine-bearing tetramer could be via iodoacetylation of the lysine epsilon amino groups and then reaction with the thiol group of the cysteine.

An example of a modification that might allow more room between tetramers would be addition of one turn of the helix to the N-terminus of the A and C helices and the C-terminus of the B and D helices. This and similar modifications would be subject to modeling and experimental constraints.

Analogous core proteins could be made as mutants of known 4-helical bundle proteins such as myohemerythrin or apoferritin, with the surface residues changed so that 8 (or more if topologically possible) lysines (2 per helix) exist on the surface for subsequent modification and attachment of the tetramer.

Poly(tetrameric) Hemoglobins with Reduced Isoelectric Points

If the isoelectric point of the whole crosslinked conjugate also affects the serum half life, via electrostatic exclusion from the renal filter's "pore", additional negative charges could be included in the crosslink itself (rather than in the hemoglobin, which could change the function of the molecule) to drop the isoelectric point of the overall crosslinked particle. An additional benefit of this might be reduced uptake by the reticuloendothelial system, this uptake being a function of pI for cationized albumin.

We have preliminary evidence from succinylation of SGE1.1 which correlates the number of modified lysines with isoelectric point. This gives a rough estimate of the number of lys to glu and/or lys to asp mutations which

5 may be necessary to reach a pI of 5 or less, the pI range
which we expect we need to significantly extend half life.
We believe that as many as 8 lysines may have to be
modified (a total shift in charge of 16 units) to drop the
10 pI roughly 2 units. It should be less disruptive of the
functional properties of hemoglobin to do this via a
eptide crosslinker rather than by mutation of the alpha
and beta globin subunits proper. However, some mutations
could be made in the crosslinker and the remainder in the
subunits. As before, the SGE1.1-cys would be attached to
15 iodoacetylated lysine epsilon amino groups by reaction at
pH 6.5-7.0.

For human serum albumin in the rat, the half life
varied roughly linearly with the pI of the protein, from
15 ca. 4.6 hours for native albumin (pI=4) to 0.8 hrs at a
pI above 9.5. Clearance was probably by multiple
mechanisms, including potentially increased uptake into
the reticuloendothelial system with increased pI. For rat
trypsinogens, the difference in serum half life between
20 versions with a pI of 5.-0 ($t_{1/2}$ of 4 min) was even larger.
Thus a lower pI clearly appears to be an important
variable in the serum half life of these proteins.

The following table gives examples of crosslinkers
between mono-cys tetramers which should diminish the
25 isoelectric point of the overall complex.

61

<u>Source</u>	<u>Sequence</u>
polyasp or polyglu	$\begin{array}{c} \text{COCH}_2\text{I} \quad \text{COCH}_2\text{I} \\ \quad \\ \text{Ac-K-(X)}_n\text{-K} \end{array}$ $n \text{ probably } \geq 10-12,$ $X = \text{D}^- \text{ or } \text{E}^-$
5 " "	$\begin{array}{c} \text{COCH}_2\text{I} \quad \text{IH}_2\text{COC} \\ \quad \\ \text{Ac-K-(G)}_n\text{-(X)}_m\text{-(G)}_n\text{-K} \end{array}$ $n > 2 \text{ to provide}$ flexibility at each terminus, $m \geq 10-12,$ $X = \text{D}^- \text{ or } \text{E}^-$
10 " "	$\begin{array}{c} \text{COCH}_2\text{I} \quad \text{COCH}_2\text{I} \\ \quad \\ \text{Ac-K-(GX)}_n\text{-K} \end{array}$ $n \geq 5-6, m \geq 10-12,$ $X = \text{D}^- \text{ or } \text{E}^-$

15 A number of the proposed crosslinkers could combine at least two, or possibly three of these attributes for potential additive effects.

It is possible that the unique amine groups in the peptide crosslinkers could be directly iodoacetylated during the peptide synthesis by treating iodoacetic acid as the last amino acid to be added, after deprotecting the lysine amine groups on the resin. In this case, the lysines would be orthogonally protected with N-FMOC or N-nitropyridinesulfonyl groups, or with BNPEOC. This could greatly simplify their synthesis.

25 Alternate methodologies to iodoacetylation as part of the synthesis could include the reaction of either sgel.1-SH or the peptide crosslinker with a heterobifunctional crosslinker specific for sulfhydryls and amines, such as sulfo-SMCC or similar reagents available from Pierce Chemical Co. (Rockford, IL).

30 Genetically Fused Poly(tetrameric) Hemoglobins

Another approach to the preparation of multimeric (e.g., polytetrameric) hemoglobin involves the genetic fusing of individual tetramers utilizing other linkers. Two or more tetramers may be linked, depending on the

desired molecular weight and the efficiency of folding of the final molecule. The dialpha (or dibeta) subunits from different tetramers of a di- alpha or di-beta Hgb might be genetically fused together into an extended polypeptide which would link the individual pseudotetrameric domains.

Proteolytically stable extended polypeptide linkages can be envisioned. Desirable linker features might include 1) a number of glycines at each end to allow flexibility in entering the dialpha (or beta) terminal domains, and to decouple the linker secondary structure from that of the dialpha (or beta) terminal domains; 2) stiffness to separate tetramers, obtainable by an extended structure such as a polyproline helix or by polyglutamate or polyaspartate; and 3) inertness to proteases (vide supra or as in a collagen sequence). Several examples of such sequences are listed below. Obviously any other of the peptide linkers mentioned in this specification could be tried after first sterically modeling the fused-dialpha (or dibeta) termini environment. The links would go from the C-terminus of one dialpha to the N- terminus of the next and would be synthesized as a single gene. Besides modeling segments of protease-resistant or negatively charged secondary structure, one or more of the Artemia linkers should be modeled between tetramers. The beta chains could also be joined in this fashion, although the results of this on protein function would be unknown. It might be feasible to make an intermolecular di-beta (sge1.1) with or without additional intrachain crosslinkages.

63

<u>Source</u>	<u>Sequence</u>
polyproline helix	di α or β C term- (G)n- (P)n- (G)n- di α or β N terminus n probably ≥ 3 , m probably $\geq 10-12$
5 polyaspartate or - glutamate	- (G)n- (D)n- (G)n- (should drop pI of complex)
10 Artemia linker (example)	- (G)n- LRRQIDLEVTGL- (G)n- ; n ≥ 0 [SEQ ID NO:2]
a helical coiled coil	- (G)n- KCAELEG (KLEALEG), <-- not fused to terminus (should form octamer with coiled-coil crosslink) [SEQ ID NO:3]
15	<p>We have determined the minimum of the intertetramer linker as follows. Two structures of human hemoglobin A₀ (either both in the oxy form or both in the deoxy form) taken or assembled from the Brookhaven Protein Data Bank were docked as close together as possible without van der Waals overlap between any residues, using the program Insight (Biosym. Inc., San Diego, CA). The distance from the alpha chain C terminal residue arg 141 to the amino terminal nitrogen of the alpha chain N terminal residue val 1 (in one structure) was then measured. This distance was ca. 22 Å when both molecules had the oxy structure and ca. 18 Å when both were in the deoxy structure. In the oxy and deoxy structures, the valine at the alpha chain N terminus is exposed at the side of a cleft in the structure, while the arg carboxylate is at the bottom of the cleft. Thus it is possible to genetically fuse these two termini without suffering a large structural displacement of residues around either terminal amino acid. A suitable intertetramer linker will be at least 18-22 angstroms long, preferably longer in order to give the structure</p>
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25	
30	
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additional flexibility. There is no fixed upper limit on the length of the linker, however, the longer the linker, the more susceptible it is to protease, and, if the molecule appears large enough, it may be phagocytosed by macrophage of the reticuloendothelial system. A few examples of suitable linkers are listed below.

An alternative fusion may be envisioned between a truncated alpha chain in one hemoglobin and the N terminal alpha val 1 in the second hemoglobin. The first molecule could be truncated at ser 138, which intermolecular N terminal to C terminal distance is about 17 Å (deoxy) and 22 Å (oxy), and examples of genetically inserted linkers spanning this distance are listed below.

Thus two hemoglobin molecules could be linked (by fusing two intermolecular alpha domains) to generate a fusion protein approximately twice the size of normal human hemoglobin. An additional intramolecular crosslink, as introduced into rHb1.1 to prevent dissociation of hemoglobin into dimers, could be included as well, giving a fusion of four alpha domains.

We expect that the genetically inserted links will be stable in the presence of proteases, due to the steric occlusion by the two hemoglobins surrounding the linkage. This resistance may be further enhanced by the use of glycines, bonds between which may be less susceptible to proteases, since most proteases have side chain specificity for residues other than glycine (which has only a hydrogen as a sidechain, and thus may result in a poor Km of this substrate for a protease). A polyproline helix may also be used as a linker to enhance stability to proteases. Fusion of a polyglutamate or polyaspartate as a linker might allow a

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much lower isoelectric point for the complex, and thus a longer serum half life.

Intertetrameric Linkers for Inclusion in
Pseudooligomeric Polypeptides

Linker	end- to- end Dista nce	conforma -tion	Comments
-(gly) ₇ -	25Å	extended	minimal length for gly linker to span termini in both oxy and deoxy structures. Longer linkers (up to 20-50 residues) may also work favorably.
5 -(gly) ₁₋₃ (ala) 12-(gly) ₁₋₃ - helix	20Å- 40Å	Ala in right handed alpha helix	the Gly are added for flexibility and minimal disturbance of Hb structure around their fusion with the N and C termni. Length is dependent on the number of glycines and the degree of extension
10 -(gly) ₁₋₃ (pro) 12-16-(gly) ₁₋₃ - proline helix	21- 48Å	pro in a left handed poly-proline helix	12, 14, 16 prolines. Length dependent on number of prolines and glycines
(gly) ₁₋₃ - (asp) ₁₋₃₀ - (gly ₁₋₃)-	26- 49Å		Asp residues add negative charges

Other residues could be substituted into these linkers while leaving their length essentially the same, including complete linkers taken from the sequence of other known human proteins such as hemoglobin, to prevent any recognition of the multimer as a foreign protein..

Use of linkers with a maximal length more than 18 Å and less than 22 Å may differentially stabilize the deoxy structure, and may result in a lowered oxygen affinity for the multimer.

10 Octameric Hemoglobins Formed Without Use of an Pseudooligomeric Globin

It is possible to produce an octameric hemoglobin, without substantial production of higher multimers, by suitable cysteine mutation of either the alpha or beta chain (see Figure 5).

Hemoglobin mutants containing one X to cys mutation in the beta chain gene (giving two per tetramer) or in the alpha chain gene (also giving two per tetramer), in which the residues mutated to cysteine are both on or very close to the surface of the subunit and are as close to the dyad axis separating the subunits, may form octamers (two hemoglobins) linked by two disulfides. Polymerization of such mutants should be retarded by the proximity of the two disulfides to each other, such that after one disulfide is formed, a third incoming hemoglobin will be sterically hindered from reacting with either free cysteine on the two original hemoglobins.

Because it is possible that this mutant may form higher order polymers (rather than simply the octamer), a diluted solution may be used in vitro for formation of disulfide bonds. The kinetics of polymerization of

hemoglobin should be at least second order (or a higher order) in hemoglobin concentration, while after one disulfide is formed, the formation of the second disulfide between two tetramers should be zero order in hemoglobin.

5 Thus the ratio of polymerized product to octamer should diminish as the hemoglobin concentration is decreased. If formation of octamers is done under oxygenated conditions, the yield of octamers vs. polymers may increase further, since the distance between the two cys mutations is less
10 in every case in the oxy hemoglobin structure than in the deoxy structure.

A list of preferred mutation sites in both the beta chain and the alpha chain is provided below:

Beta and alpha chain mutation sites for x to cys mutations to form disulfide-bond linked octameric hemoglobin.

	Chain/ Mutation	Old Distance(Å)	New Distance(Å)	Comment
5	beta Asn 80 to cys	22	18	no listed deleterious mutations, asn 80 is on surface
	beta Asp 70 to cys	24	22	Hb Tampa ^a (asp to tyr) has no major abnormal property listed; Hb G-His-Tsou (asp to gly) has increased O ₂ affinity; is on surface
10	alpha Asn 78 to cys	24	20	on surface; no major ^a abnormal properties of known mutations of asn 78
	alpha Asp 75 to cys	22A	18	on surface; no major abnormal properties of known mutations of asp 75
	alpha Asp 74 to cys	26	20	on surface; no major ^a abnormal properties of known mutations of asp 74

15 ^a R.N. Wrightstone. Policies of the International Hemoglobin Information Center (IHIC), Comprehensive Sickle Cell Center, Medical College of Georgia. 1988.

Gene Construction and Expression

The DNA sequences encoding the individual polypeptide chains may be of genomic, cDNA and synthetic origin, or a combination thereof. Since the genomic globin genes contains introns, genomic DNA must either be expressed in a host which can properly splice the premessenger RNA or modified by excising the introns. Use of an at least partially synthetic gene is preferable for several reasons. First, the codons encoding the desired amino acids may be selected with a view to providing unique or nearly unique restriction sites at convenient points in the sequence, thus facilitating rapid alteration of the sequence by cassette mutagenesis. Second, the codon selection may be made to optimize expression in a selected host. For codon preferences in E. coli, see Konigsberg, et al., PNAS, 80:687-91 (1983). Finally, secondary structures formed by the messenger RNA transcript may interfere with transcription or translation. If so, these secondary structures may be eliminated by altering the codon selections.

Of course, if a linker is used to genetically crosslink subunits, the linker will normally be encoded by a synthetic DNA.

The present invention is not limited to the use of any particular host cell, vector, or promoter. The host cell may be prokaryotic or eukaryotic, and, in the latter case, may be a plant, insect or mammalian (including human) cell. The cell may also be of any suitable tissue type, including, inter alia, an erythrocyte. However, the preferred host cells are bacterial (especially, E. coli) and yeast (especially S. cerevisiae) cells. The promoter selected must be functional in the desired host cells. It preferably is an inducible promoter which, upon induction,

provides a high rate of transcription. A preferred bacterial promoter is the Tac promoter, a trp/lac hybrid described fully in DeBoer, U.S. 4,551,433 and commercially available from Pharmacia-LKB. Other promoters which might be used include the temperature sensitive lambda P_L and P_R promoters, as well as the lac, trp, trc, pIN (lipoprotein promoter and lac operator hybrid), gal and heat shock promoters. The promoter used need not be identical to any naturally-occurring promoter. Guidance for the design of promoters is provided by studies of promoter structure such as that of Harley and Reynolds, Nucleic Acids Res., 15:2343-61 (1987) and papers cited therein. The location of the promoter relative to the first structural gene may be optimized. See Roberts, et al., PNAS (USA), 76:760-4 (1979). The use of a single promoter is favored. Suitable yeast expression systems are described in detail elsewhere in this specification.

The vector used must be one having an origin of replication which is functional in the host cell. It desirably also has unique restriction sites for insertion of the globin genes and the desired regulatory elements and a conventional selectable marker. A vector may be modified to introduce or eliminate restriction sites to make it more suitable for further manipulations.

The component polypeptide chains of the multimeric hemoglobin may be expressed either directly or as part of fusion proteins. When expressed as fusion proteins, the latter may include a site at which they may be cleaved to release the globin-related moiety free of extraneous polypeptide. If so, a site sensitive to the enzyme Factor Xa may be provided, as taught in Nagai and Thorgenson, EP Appl 161,937, incorporated by references herein. Alternatively, the fusion proteins may be synthesized, folded and heme incorporated to yield a hemoglobin

analogue. The direct expression of the component polypeptides is desirable.

5 In bacterial mRNA, the site at which the ribosome binds to the messenger is a polypurine stretch which lies 4-7 bases upstream of the start (AUG) codon. The consensus sequence of this stretch is 5'...AGGAGG...3', and is frequently referred to as the Shine-Dalgarno sequence. Shine and Dalgarno, Nature, 254: 34 (1975). The exact distance between the SD sequence and the translational start codon, and the base sequence of this "spacer" region, affect the efficiency of translation and may be optimized empirically. Shepard, et al., DNA 1: 125 (1985); DeBoer, et al., DNA 2: 231 (1983); Hui, et al., EMBO J., 3: 623 (1984).

15 In addition, the SD sequence may itself be modified to alter expression. Hui and DeBoer, PNAS (USA), 84:4762-66 (1987). Comparative studies of ribosomal binding sites, such as the study of Scherer, et al., Nucleic Acids Res., 8:3895-3907 (1980), may provide guidance as to suitable base changes. If the hemoglobin is to be expressed in a host other than E. coli, a ribosomal-binding site preferred by that host should be provided. Zaghibil and Doi, J. Bacteriol., 168:1033-35 (1986).

25 Any host may be used which recognizes the selected promoter and ribosomal binding site and which has the capability of synthesizing and incorporating heme. Bacterial and yeast hosts are preferred.

30 The intracellularly assembled hemoglobin may be recovered from the producing cells and purified by any art-recognized technique.

Polycistronic Expression in Bacteria

While not required, it is desirable that the subunits, when expressed in bacteria, be co-expressed in the same

cell, and it is still more preferable that they be co-expressed polycistronically. A polycistronic operon encodes a single messenger RNA transcript having one promoter sequence, but two or more pairs of start and stop codons that define distinctly translatable sequences. Each such sequence is known as a "cistron," and the polypeptides corresponding to the cistrons are thus co-expressed under the control of the single promoter.

The majority of bacterial operons are polycistronic, that is, several different genes are transcribed as a single message from their operons. Examples include the lactose operon with three linked genes (lacZ, lacY and lacA) and the tryptophan operon with five associated genes (trpE, trpD, trpC, trpB, and trpA). In these operons, the synthesis of messenger RNA is initiated at the promoter and, within the transcript, coding regions are separated by intercistronic regions of various lengths. (An operon is a cluster of genes that is controlled as a single transcriptional genetic unit). Translational efficiency varies from cistron to cistron. Kastelein, et al., Gene, 23: 245-54 (1983).

When intercistronic regions are longer than the span of the ribosome (about 35 bases), dissociation at the stop codon of one cistron is followed by independent initiation at the next cistron. With shorter intercistronic regions, or with overlapping cistrons, the 30S subunit of a terminating ribosome may fail to dissociate from the polycistronic mRNA, being instantly attracted to the next translational initiation site. Lewin, Gene Expression, 143-148 (John Wiley & Sons: 1977).

Unlike bacterial mRNAs, eukaryotic mRNAs are generally monocistronic in nature. Lewin, Gene Expression, 157.

In one embodiment, expression of the genes encoding two or more component polypeptides are driven by a single promoter, and the genes are arranged so that a polycistronic messenger RNA transcript is transcribed, from which the separate globin-like polypeptides are subsequently translated. However, the present invention includes the co-expression of the different polypeptides from separate promoters, i.e., the host transcribes separate alpha and beta globin mRNAs.

Ideally, alpha and beta globin-like domains are expressed in stoichiometrically equal amounts. While use of a single promoter does not guarantee equality, it eliminates one unbalancing influence --differences in transcription owing to differences in promoter strength and accessibility. If differences in promoter strength were minimized by use of two identical promoters on the same plasmid, plasmid stability would be reduced as there would be a propensity toward recombination of the homologous regions.

Preferably, the alpha and beta globin-like domain-encoding genes are arranged so that the ribosome will translate the alpha globin cistrons first. The rationale is that there is some basis for believing that alpha globin affects the folding of beta globin. Nonetheless, the position of the genes may be switched so that a beta globin-like domain is synthesized first.

The stability of the polycistronic mRNA transcript, the efficacy of its translation into alpha and beta globin-like polypeptides, and the folding of the globin chains into tetrameric hemoglobin may be modified by varying the length and base sequence of the intercistronic regions (the region lying between the stop codon of one cistron and the start codon of the next cistron), the phasing of a second cistron relative to a first cistron,

and the position and sequence of the ribosomal binding site for the one cistron relative to the preceding cistron.

In a preferred embodiment, the alpha and beta globin-like polypeptides genes are each preceded by a short "introductory" cistron or "ribosomal loader" which facilitates the subsequent translation of the globin cistron. In Figure 2, region A contains two cistrons and a Shine-Delgarno sequence preceeding each cistron. The first Shine-Delgarno sequence (SD#1) is bound by the ribosome, which then translates the first cistron, a short cistron encoding an octapeptide. (This cistron is referred to as an "introductory cistron or ribosomal loader.") The second cistron is a globin gene, in this case, an FX alpha-globin gene. The Shine-Delgarno sequence (SD#2) for facilitating translation of the second cistron actually lies within the first cistron. For this reason, the two are said to be "translationally coupled". Region B is identical in structure, except that the second cistron encodes FX-beta globin. Between regions A and B is a 43-base intercistronic region. The introductory cistrons of regions A and B correspond to the first cistron of the two-cistron expression system denoted pCZ144 in Schoner, et al., Meth. Enzymol., 153: 401-16 (1987). The present invention is not, however, limited to the particular "starter" cistron taught by Schoner, et al.; other introductory cistrons that allow for restart of high level translation of a following cistron may be used.

Guidance as to the design of intercistronic sequences and as to the location of SD sequences may be obtained by comparing the translational efficiency of spontaneous or controlled mutants of the same polycistronic operon, as exemplified by Schoner, et al., PNAS, 83: 8506-10 (1980). It is also possible to look for consensus features in the

intercistronic regions of different operons. McCarthy, et al., EMBO J., 4: 519-26 (1985) have identified a translation- enhancing intercistronic sequence in the E. coli atp operon.

5 The present invention is intended to reduce or avoid the localization of the hemoglobin or its component polypeptides into inclusion bodies. Consequently, a further feature of the invention is that the functional hemoglobin is substantially found (preferably over 80%) in
10 the soluble fraction of the cell. It appears that with this invention, over 90% of the functional hemoglobin can be so directed when alpha₂ beta₂ hemoglobin is assembled from alpha- and beta- globin chains co-expressed from a tetracistronic operon as described herein. With di-alpha,
15 beta₂ hemoglobin, nearly 100% is soluble when expression is induced at 25°C and less at higher induction temperatures. These percentages reflect the percent of all di-alpha and beta chains found in the soluble fraction of the cell and not actual recovery of protein from the cell.

20 *Expression in Yeast*

 In another embodiment the present invention relates to the production of hemoglobin-like molecules in yeast. Our preferred host for expression in yeast is Saccharomyces cerevisiae. However, other fungi or yeast may be used for
25 the purpose, such as strains of Aspergillus or Pichia. For yeast to be a suitable host it must be capable of being transformed with recombinant vectors, either replicating or integrating types. This allows the insertion of the desired DNA sequence for the gene of
30 interest. It must also be capable of high density cell growth, in appropriate volume to provide sufficient cell mass to isolate the desired gene product from the desired

reaction vessels, where ideally the growth would be easily controlled by several parameters including nutrient formulation, agitation and oxygen transfer and temperature. It is also desirable to be able to induce the expression of protein synthesis with the manipulation of the media, temperature, or by the addition or consumption of certain chemicals. Finally, to be a suitable host, the yeast must be capable of producing recombinant proteins, preferably in excess of 1% of the total cell protein. This allows more facile isolation of the desired recombinant protein.

Either a haploid or a diploid strain of S. cerevisiae may be used. For example, the following diploid strains are preferred:

15 BJY3505 x RSY330
 BJY3505 x BJY 1991

Other matings may likewise be used in practicing the present invention.

20 The use of protease-deficient strains may also be advantageous.

Yeast expression systems can be divided into two main categories: (1) Systems designed to secrete protein and (2) system designed for the cytoplasmic expression of proteins. At present, cytoplasmic expression is preferred since the yeast cells fold together the globin chains and incorporate heme to produce hemoglobin in vivo. However, it is possible to separately express and secrete the alpha and beta globin chains and assemble hemoglobin in vitro.

30 The globin genes must be placed under the control of a suitable promoter. The commonly used yeast promoters generally fall into two broad categories: regulated and constitutive. Constitutive promoters that are in wide use include GAP, PGK (phosphoglycerate kinase) and the α -

factor promoter. Regulated promoters have also been used and these include the yeast metallothionein promoter (regulated by copper), the Gal1-10 promoter, GAL7 promoter (regulated by galactose and glucose) the ADHII promoter (regulated by ethanol and glucose) the PH05 promoter (phosphate regulation) and several hybrid promoters such as PH05-GAP, GAL-PGK, ADHII-GAP, and GAL-CYC1.

The use of a GAL-GAP hybrid promoter is preferred. Both elements (the GAL_{UAS} and the GAP transcriptional initiation site) are well understood. Studies on the mechanisms of transcriptional regulation of the GAL regulon have been fairly extensive. The galactose regulon includes five genes that encode enzymes required for the utilization of galactose. Four of these genes (GAL1, GAL7, GAL10, and GAL2) are expressed only in the presence of galactose. Galactose induction does not occur in the presence of glucose unless the yeast strain bears a mutation in the REG1 gene. The GAL1, 7, 10 and 2 genes are regulated by at least two other genes, GAL80 and GAL4. The GAL4 gene is a transcriptional activator protein that activates mRNA synthesis from the GAL1, 7, 10 and 2 upstream activator sequences (UAS_{GAL}). Although GAL4 is constitutively expressed, it is functionally silent in the absence of galactose. Repression of GAL4 activity, in the absence of galactose is maintained by the product of the GAL80 gene. The GAL80 protein apparently interacts physically with GAL4 to prevent transcriptional activation. Presumably galactose or a galactose derivative prevents this interaction to allow GAL4 mediated induction.

Haploid strains of *S.cerevisiae* have three different genes encoding the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAP). These genes have been designated

TDH1, TDH2 and TDH3 and each is present as a single copy per haploid genome. The TDH3 gene produces approximately 60% of the cell's GAP enzyme and TDH1 and 2 produce about 12% and 28%, respectively (McAllister, L and M.J. Holland, 1985. J. Biol Chem, 260: 15019-15027). Holland's group (Holland et al. 1981. J. Biol Chem, 256:1385-1395; and Holland et al. 1983. J Biol Chem 258:5291-5299) has cloned and characterized the three GAP genes of *S.cerevisiae*. The clones have been designated pGAP11, pGAP63, and pGAP491. pGAP491 corresponds to the TDH3 gene and is therefore, the most highly expressed.

This promoter is commonly used as a 600-850bp fragment and is essentially un-regulated. In its long form this is a very powerful promoter. The form we are using consists of only ~200bp 5' of the translational initiation site. This form, with no added enhancer sequences is substantially less active than the longer form of the promoter (Edens, L. et al. Cell, 37:629 (1984)). Our addition of the GAL enhancer region confers both regulation and high levels of expression. With only the GAP491 promoter, alpha and beta globin were produced at a level of less than 0.2% total cell protein; with the GAL-GAP491 hybrid promoter, expression jumped to 7-10% total cell protein.

Several other hybrid promoters are of particular interest: GAL-SIGMA; SIGMA-GAP; GAL-EF III; SIGMA-EF III.

One could easily conceive of other promoter systems that would also work. This would include, but not be limited to, a variety of constitutive promoters. For example, the yeast mating factor α (MF α) promoter or the mating factor a promoter MF(a), the phosphoglycerate kinase promoter (PGK), hexokinase1, hexokinase2,

glucokinase, pyruvate kinase, triose phosphate isomerase, phosphoglycerate isomerase, phosphoglycerate mutase, phosphofructose kinase or aldolase promoters may all be used. In short, any well expressed yeast promoter may work for expression of hemoglobin in yeast. A wide variety of naturally occurring, regulated promoters could also be used, for example: GAL1-10, GAL7, PH05, ADHII have all been used to produce heterologous proteins in yeast. A variety of synthetic or semi-synthetic yeast promoters could also be employed such as GAL-PGK, GAL-MF α -1, GAL-MFa1, GAL-SIGMA. ADHII regulatory sequences could also be coupled to strong transcriptional initiation sites derived from a variety of promoters. The PH05 regulatory sequence or the sigma element regulatory sequences could also be used to construct powerful hybrid promoters. In addition to yeast promoters, it is conceivable that one could use a powerful prokaryotic promoter like the T7 promoter. In this case, one could place the T7 polymerase under the control of a tightly regulated yeast promoter. Induction of the phage polymerase in yeast cells bearing hemoglobin genes under T7 promoter regulation would allow transcription of the genes by this very efficient phage polymerase.

Because most of the yeast regulatory sequences described above serve as targets for proteins that are positive regulators of transcription, it is conceivable that these proteins may limit transcription in situations where the target sequence is present in many copies. Such a situation may obtain with vectors such as pC1B, pCIT, pC1U or pC1N which may be present in excess of 200 copies per cell. Over-expression of the positive regulator (for example GAL4) may result in enhanced expression. It is possible to construct a strain in which the GAL4 gene is altered to remove its promoter and the promoter replaced

with the GAL7 or GAL1-10 promoters, both of which are transcribed more efficiently than the GAL4 promoter. In this situation, the positive transcriptional activator protein GAL4 would be expressed at elevated level at the time hemoglobin expression was induced.

5 The consensus sequence for higher eukaryotic ribosome binding sites has been defined by Kozack (Cell, 44:283-292 (1986)) to be: G^{AA}₆CCAUGG (SEQ ID NO:10). Deviations from this sequences, particularly at the -3 position (A or G),
10 have a large effect on translation of a particular mRNA. Virtually all highly expressed mammalian genes use this sequence. Highly expressed yeast mRNAs, on the other hand, differ from this sequence and instead use the sequence AAAAAUGU (Cigan and Donahue, Gene, 59:1-18
15 (1987)). The ribosome binding site that we use for expression of the α and β -globins corresponds to the higher eukaryotic ribosome binding site. It is within the contemplation of this invention to systematically alter this RBS to test the effects of changes that make it more
20 closely resemble the RBS of yeast. It should be pointed out, however, that alterations at the -2, -1 and +3 positions, in general, have been found to only slightly affect translational efficiency in yeast and in mammals.

Intracellular expression of genes in S. cerevisiae is
25 primarily affected by the strength of the promoter associated with the gene, the plasmid copy number (for plasmid-borne genes), the transcription terminator, the host strain, and the codon preference pattern of the gene. When secretion of the gene product is desired, the
30 secretion leader sequence becomes significant. It should be noted that with multicopy plasmids, secretion efficiency may be reduced by strong promoter constructions. Ernst, DNA 5:483-491 (1986).

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A variety of extrachromosomally replicating vectors (plasmids) are available for transforming yeast cells. The most useful multicopy extrachromosomal yeast vectors are shuttle vectors that use a full length 2μ -circle combined with an E. coli plasmid. These vectors carry genes that allows one to maintain the plasmid in appropriate yeast mutants and antibiotic resistance markers that allow selection in E. coli. Use of the full-length 2μ -circle, in contrast to vectors containing only a partial 2μ sequence, generally results in much higher plasmid stability, particularly in yeast strains that have been cured of endogenous 2μ plasmid. The pC series of vectors described herein are vectors of this type.

Strains could also be constructed in such a way that the GALGAP hemoglobin expression cassettes were integrated into chromosomes by using yeast integrating vectors. Although the copy number of the hemoglobin genes would be lower than for plasmid vectors, they would be quite stable and perhaps not require selection to be maintained in the host cell. Yeast integrating vectors include Yip5 (Struhl, et al, PNAS, 76:1035- 39, 1989), Yip1 (Id.), and pGT6 (Tchumper and Carbon, Gene, 10:157-166, 1980). For information on these and other yeast vectors, see Pouwels, et al., Cloning Vector, VI-I, et seq. (Elsevier, 1985).

The genes encoding the desired globin-like domains may be introduced by separate plasmids, or both upon the same plasmid.

Highly expressed yeast genes show a very high codon bias. The genes encoding glyceraldehyde-3-phosphate dehydrogenase and ADH-I, for example, show a 90% bias for a set of 25 codons. Highly expressed yeast genes (>1% of the total mRNA) have yeast codon bias indices of >.90. Moderately expressed genes (0.1-.05% of the total mRNA) have bias indices of 0.6-0.8, and genes expressed at low

levels (>0.05% of the total cell protein) have a codon bias of 0.10-0.50 (Bennetzen and Hall, J. Biol. Chem., 257:3026-3031 (1982)). The calculated value for the codons of the human α -globin cDNA is 0.23. A similar value can be calculated for the β -globin cDNA. Because there is a very high correlation between the most commonly used codons, it is possible that hemoglobin expression from the human cDNA in yeast may be limited by the availability of the appropriate tRNA molecules. If this is so, a complete synthesis of the gene using the most highly favored yeast codons could improve the expression levels. It is quite possible that the greatest negative effect of adverse codon use would be if there was an abundance of codons used in the cDNA that are represented by low abundance tRNAs. In such a case, high level expression of hemoglobin could completely drain that pool of tRNA molecules, reducing translation not only of hemoglobin but of yeast proteins that happen to use that codon as well. In the case of the α -globin human cDNA, the most commonly used leucine codon is CTG (14 of 21), this codon is never used in highly expressed yeast genes (Guthrie and Abelson, The Molecular Biology of the Yeast Saccharomyces, Eds. Stratern, Jones and Broach, 1982. Cold Spring Harbor, NY). The low codon bias index and the presence of rare yeast codons in the globin cDNAs have been sufficient incentive for us to synthesize a modified form of the alpha- and beta-globin genes using the preferred yeast codons.

Miscellaneous

The appended claims are hereby incorporated by reference as a further enumeration of the preferred embodiments. All cited references are incorporated by

reference to the extent necessary to enable the practice of the invention as now or hereafter claimed.

Preparation of expression vectors suitable for use in production of the claimed multimeric hemoglobins may be facilitated by the Budapest Treaty deposit of the following vectors, all made with American Type Culture Collection, Rockville, Maryland USA on May 10, 1990:

ATCC 68323 pDL III-14c

This is a derivative of pKK223-3 (Pharmacia LKB, Piscataway, New Jersey, USA) and pGEM1 (PromegaCorp., Madison, Wisconsin, USA) which carries synthetic genes for des-Val alpha globin and des-Val beta globin as part of a polycistronic operon driven by a single Tac promoter.

ATCC 68324 pDL IV-8a

This is a derivative of PDL III-14c which contains a fused gene encoding an alpha globin moiety, a glycine, and a second alpha globin moiety, as well as a second gene encoding des-Val beta globin.

ATCC 20992 pGS 389

This is a yeast vector which expresses alpha and beta globin under control of GALGAP promoters.

The deposit of these vectors should not be construed as a license to make, use or sell them.

Example 1:Construction of di- α globin mono-cysteine (A71C, D75C, or S81C) mutant expression vector

5 The following plasmids, whose preparation is fully described in Hoffman, et al., WO88/09179, were manipulated in this Example.

Plasmid pDL II-83a

10 A gene encoding a Met initiation codon, a Factor X site (Ile-Glu-Gly-Arg) (SEQ ID NO:11), and human alpha globin, collectively referred to as FX-A, was synthesized and cloned into the XmaI/PstI sites of M13mp19. The EcoRI-PstI fragment bearing the FX-A gene was excised and recloned into pKK 223-3, placing it under control of the Tac promoter. This derivative was called pDLII-62m. The
15 FX-A gene was removed from pDLII-62m and ligated with EcoRI/PstI linearized pGEM1, forming pGEM-FX-A. This was digested with NdeI and EaqI, removing the Factor Xa coding sequence (and part of the α globin coding sequence). The excised fragment was replaced by a synthetic
20 oligonucleotide which restored the missing α globin codons; the resulting plasmid was named pDLII-83a. The protein expressed was "Met-Val-Leu-...."

25 *Plasmid pDLII-91f*, in which the gene encodes "Met Leu..." instead of "Met-Val-Leu", was likewise constructed from pGEM-FX-A, but with a different synthetic replacement oligonucleotide, missing the Val codon.

Plasmid pSGE 1.1 E4

30 This plasmid (also known as SGE1.1) is depicted in Figure 1. Plasmid pDLIV-8A may be converted to SGE1.1E4 by the following protocol.

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The expression plasmid pDLIV-8a contains the dialpha coding sequences, in which the alpha globins are linked by a single glycine codon, and the des-val beta globin genes, under the control of a single Ptac promoter. The plasmid encodes ampicillin resistance, does not have a functional tetracycline resistance gene, and is Rop+ and Lac. Oligonucleotide directed site specific mutagenesis using a commercially available kit such as DoubleTake™ (Stratagene, Inc.) can be used to insert the Presbyterian mutation into the beta globin sequence.

AAC		AAA
TTG	→	TTT
asn ¹⁰⁸		lys

The final expression plasmid, SGE 1.1E4 (amp R, tet R, Rop-, Lac+) is then constructed by insertion of both a functional tetracycline resistance gene and the lacI gene which encodes the lac repressor protein that inhibits the Ptac promoter until induction with an inducing agent. These modifications are described below.

The initial modification to the plasmid is the insertion of the lacI gene. This gene can be synthesized by polymerase chain reaction (PCR) amplification, according to the manufacturer's protocol (Perkin Elmer Cetus, Norwalk, CT) using the F episome of E. coli strain JM109 (FtraD36, proAB, lacI^ΔM15) as a substrate. The following oligonucleotides can be used as primers:

Forward:

5' GCGGCCGCGGAAGAGTCAATTCAGGAGGGTG 3' (SEQ ID NO:12)

Reverse:

5' GCGGCCGTCACTGCCCCGCTTTCCAGTCGGGAA 3' (SEQ ID NO:13)

The primers contain, at their 5' ends, sequences which encode for NotI restriction enzyme sites. The product of the PCR amplification reaction can be blunt ended and cloned into the PuvII site of the expression plasmid. The PuvII site is not reconstructed during this cloning step, so digestion with PuvII following ligation will linearize plasmids which do not incorporate the lacI sequence. Linearized plasmids will not transform E. coli. Because the primers are complementary only to the translated portion of the lacI gene, this fragment does not contain its own promoter. Note that inducibility or expression of hemoglobin is dependent on the orientation of the lacI gene, thus orientation should be checked after insertion of the lacI gene. The correct orientation has a smaller EcoR5 fragment than the incorrect orientation. Moreover, insertion of the lacI repressor gene into the PuvII restriction site inactivates the rop gene product and results in increased plasmid copy number.

The final modification to the plasmid is restoration of a functional tetracycline resistance gene. This can be accomplished by digestion of commercially available pBR322 with EcoRI followed by insertion via ligation of a synthetic DNA linker containing the 5' and 3' ends complementary to the EcoRI overhangs and an internal BamHI site. The BamHI fragment from this modified pBR322 vector containing the 5' coding sequence of the tetracycline resistance gene is purified by agarose gel electrophoresis, then inserted into the BamHI site of the modified pDL IV-8a plasmid. Only one orientation of the tet^R fragment results in tetracycline resistance; strains can be screened for the proper orientation by growth on the appropriate medium. Insertion of the ter^R fragment

into the modified vector restores tetracycline resistance and produces SGE1.1E4.

Plasmid pGEM di-alpha.

5 The di-alpha gene-bearing SmaI/PstI fragment of SGE 1.1 E4 was ligated with SmaI/PstI-cut pGEM 1 to form pGEM di-alpha.

1.1 Subcloning of the α gene into phagescript

10 The desfx α pGem (pDLII-83a) vector was cut with EcoRI and PstI endonucleases and ligated into EcoRI/PstI digested phagescript (obtained from Stratagene). E. coli strain DH5 α was transformed with the ligation mixture and cells were plated on 2xYT plates overlaid with 3 ml top agar containing 10 μ l 100 mM IPTG, 25 μ l 2% X-Gal in DMSO and 15 150 μ l XL-1 cells (Stratagene). Clear plaques were picked and grown at 37°C in 2xYT containing XL-1 cells. Double stranded DNA was isolated from the cultures and checked for the presence of the 500 bp α gene by restriction analysis and agarose gel electrophoresis. Single stranded DNA was isolated from one of the desfx α phagescript 20 transformants (named f191). The single stranded DNA was sequenced to confirm the presence of the desfx α gene in the phagescript.

1.2 Mutagenic Oligonucleotides

25 Three mutagenic oligonucleotides were used in three separate mutagenic reactions. The sequences of the oligonucleotides were as follows (mutant codon is underlined):

Nigeria mutation: α S81C

5' CCG AAC GCG TTG TGC GCT CTG TCT GAT 3'

30 [SEQ ID NO:14]

α D75C

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